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THE INFLUENCE OF ABDOMINAL STRETCH ON SALIVARY GLAND DEGENERATION IN THE TICK *Amblyomma hebraeum* KOCH (ACARI: IXODIDAE)

by

DONELDA L. PATRIQUIN

(C)

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE INFLUENCE OF ABDOMINAL STRETCH ON SALIVARY GLAND DEGENERATION IN THE TICK *AMBLYOMMA HEBRAEUM* KOCH (ACARI: IXODIDAE) submitted by DONELDA LOUISE PATRIQUIN in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in ZOOLOGY.

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ABSTRACT

Female ixodid ticks take a large bloodmeal in preparation for egglaying. Within four days of the bloodmeal, the salivary glands degenerate, a process mediated by a hormone, 'tick salivary gland degeneration factor' (TSGDF); TSGDF is probably an ecdysteroid. Degeneration is characterized by a decrease in fluid secretory competence and the appearance of autophagic vacuoles in the fluid secretory labyrinth of the type III acinus.

Artificial stretch of the abdomen of small ticks by infusing liquids or compressed nitrogen gas was attempted to investigate the hypothesis that abdominal stretch is the stimulus for the release of TSGDF and salivary gland degeneration. Ticks were infused over 24 hours with isosmotic solutions or with nitrogen gas, and then measured for hemolymph ecdysteroid titer and fluid secretory competence of the glands after five days incubation. When compared with a number of controls, there was no instance in which salivary gland degeneration was correlated to stretch of the abdomen. The question of the sensory stimulus for salivary gland degeneration thus remains open.

The tick, *Hyalomma anatolicum excavatum*, undergoes a seasonal diapause during the autumn and winter, delaying oviposition for several months following the bloodmeal. Unpublished results of Kaufman had suggested that salivary gland degeneration was also delayed. To further test the hypothesis that TSGDF is an ecdysteroid, a number of ticks were fed to engorgment during the months of September and October of 1989. The ticks were sampled over the next four months, including the early days of oviposition. In each case, fluid secretory competence and hemolymph ecdysteroid titers were measured. Although oviposition did not commence until 100 days following engorgement, salivary gland degeneration seemed to be advanced by day 17 (the earliest time sampled), and hemolymph ecdysteroid titer was low (about 7 ng/mL 20-OH-E equivalents). Due to the limited sample size in this experiment, I could not exclude the possibility that ecdysteroid titers had risen early in the postengorgement period, triggered salivary gland

degeneration and then had declined to low levels by day 17.

- Finally, small partly fed ticks, when forcibly removed from the host, normally reattach if given the opportunity. I tested whether high ecdysteroid titers might be responsible for inhibiting the reattachment of large partially-fed ticks: Small partly fed ticks were removed from the host, and infused over 24 hours with large amounts of 20-OHE. Of the ticks which survived, 15% reattached, compared to 44% of the controls. The few ticks which did reattach had low hemolymph ecdysteroid titers and high salivary fluid secretory rates, suggesting that high hemolymph ecdysteroid levels do inhibit reattachment to the host.

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CHAPTER ONE

General Introduction

Biology of the Tick

The ixodid ticks (Acari: Ixodidae) are all blood-sucking arthropods. The female takes a huge single blood meal, and then detaches in preparation for oviposition (reviewed by Balashov, 1972; Kaufman, 1983). The male can undergo several feeding cycles, but takes only very small blood meals relative to the female.

Feeding in the female tick is divided into two phases: an initial slow phase which takes 6 to 9 days, by which time the tick has reached approximately ten times the unfed weight, and a final rapid stage which takes 12 to 24 hours, by which time the tick has attained about 100 times the unfed weight and detaches from the host (Lees, 1946). In hematophagous insects, the meal is of much shorter duration (usually a few minutes) and excess fluid is excreted by the Malpighian tubules (Maddrell, 1963). The blood meal is concentrated in female ticks by periodic secretion of the excess fluid by the salivary glands into the host during the feeding cycle, maintaining the hemolymph hyperosmotic to the bloodmeal (Tatchell, 1967, 1969; Kaufman and Phillips, 1973).

Due to their important role in osmoregulation and several other functions, the salivary glands are very prominent organs in ticks. The paired glands, consisting of "grapelike" clusters of acini along a duct system, extend along the body wall from the oral cavity to just past the last leg. The salivary glands consist of three types of acini along a common duct system. Type I acini, found on the anterior section of the main salivary duct, are believed to function in a water vapor uptake mechanism used by ticks during periods of dehydration while off the host (Knülle and Devine, 1972; Kahl, *et al*, 1990). The Type II acini, found on the proximal lobular ducts (branching off the main duct), may contribute to the attachment cement secretion used to anchor the tick to the host. Type III acini, which are on the distal portions of the lobular ducts, are also thought to be responsible for cement secretion (Balashov, 1972; Chinery 1973; Walker, *et al*,

1985). As feeding progresses, specialized cells ('f-cells' and 'ablumenal interstitial cells'), in these acini form an interdigitating 'secretory labyrinth' thought to be the major site of fluid secretion (Fawcett, *et al*, 1981).

Salivary Gland Degeneration

A number of critical physiological events occur during feeding. During the slow phase of feeding, the abdominal cuticle unfolds and stretches and additional endocuticle is secreted to accommodate the increased volume of the bloodmeal (Lees, 1952). Immediately after engorgement, the tick initiates the final phase of vitellogenesis (Balashov, 1972; Diehl, *et al*, 1982a). The salivary glands also degenerate within a few days of engorgement (Till, 1961).

Salivary gland degeneration depends on two factors: (1) feeding past a species specific 'critical weight', above which degeneration occurs, and (2) mating. Degeneration is characterized by a decrease in fluid secretory competence and autophagic vacuole formation in the secretory labyrinth of the type III acinus (Harris and Kaufman, 1981). Degeneration of the salivary glands is a hormonally-regulated process. The salivary glands of partially fed females under the critical weight, transplanted into replete females, degenerate in a manner similar to the intact glands of the recipient female. The hormone discovered by these experiments was named 'tick salivary gland degeneration factor' (TSGDF; Harris and Kaufman, 1981). Ecdysteroids induce degeneration *in vivo* and *in vitro* (Harris and Kaufman, 1985) and hemolymph ecdysteroid titers increase approximately 30-fold following engorgement (Connat, *et al*, 1985; Kaufman, 1991), suggesting that TSGDF is an ecdysteroid.

As mentioned above, the degeneration process is triggered by feeding past a species-specific critical weight. Partially fed ticks, removed from the host after feeding to just below the critical weight (about 300 mg for *A. hebraeum*; Harris and Kaufman, 1984), do not form autophagic vacuoles in the Type III acini after four days off the host. They do show a 75% decrease in salivary gland secretory competence, however. Ticks feeding to

weights above the critical weight, lose virtually all secretory competence four days after removal from the host, and autophagic vacuoles form in the secretory labyrinth of the Type III acini (Harris and Kaufman, 1981, 1984). The relationship between a 'critical weight' and salivary gland degeneration suggests that stretching of the abdomen could be a stimulus for release of TSGDF, just as it is a stimulus for the release of moulting and diuretic hormones in insects (Wigglesworth, 1956, Maddrell, 1964 a,b, Nijhout, 1979).

The Study

For this study my primary goal was to further investigate ecdysteroids as a candidate for TSGDF. The main project was to test whether abdominal stretch, normally caused by feeding, is a factor initiating salivary gland degeneration in ticks. By infusing ticks over 24 hours with isosmotic solutions, I attempted to bring the weight of small partially fed ticks to a point as far above the critical weight as possible. I then examined these ticks 5 days following removal from the infusion apparatus for loss of fluid secretory competence and increase in hemolymph ecdysteroid concentration. If abdominal stretch does induce release of TSGDF, one would expect to correlate loss of fluid secretory competence and an increase in hemolymph ecdysteroid titer to stretch above that seen at critical weight.

Testing whether abdominal stretch was a factor initiating degeneration was complicated by the fact that salivary glands of ticks below the critical weight do not suffer tissue degeneration with time off the host, but do show a considerably reduced fluid secretory competence (Kaufman, 1983, Harris and Kaufman, 1984). This necessitated the use of strict controls to distinguish between true tissue degeneration and mere loss of fluid secretory competence. These controls included: (1) very small ticks infused to below the critical weight, controlling for the effects of infusion, and (2) normally fed ticks (a) above the critical weight, (b) just below the critical weight, and (c) well below the critical weight, corresponding to the starting and infused weights of the experimental ticks.

Secondly, using a species of tick (*Hyalomma anatolicum excavatum*) which undergoes a seasonal diapause after engorgement to delay vitellogenesis and oviposition, I examined the levels of hemolymph ecdysteroids and degree of salivary gland degeneration during diapause and oviposition. With the hypothesis that TSGDF is an ecdysteroid, I examined whether salivary gland degeneration was inhibited during diapause, and if this was correlated with low ecdysteroid titer. Engorged ticks were sampled periodically during diapause and during egg-laying to investigate the timing of salivary gland degeneration relative to change in hemolymph ecdysteroid titer.

Finally, partially-fed ticks, below critical weight, can normally reattach to the host and complete the blood meal, while larger ticks do not. It is possible that the rising hemolymph ecdysteroid titer in ticks above the critical weight, in addition to triggering degeneration of the salivary glands, is a factor inhibiting reattachment to the host. In the last section of my thesis, I tested whether high hemolymph ecdysteroid titer prevents reattachment and feeding to engorgement.

CHAPTER TWO

Stretch of the Abdomen by Liquid Infusion

Introduction

Within four days of engorgement, the salivary glands of ixodid ticks degenerate, a process characterized by a decrease in fluid secretory competence and formation of autophagic vacuoles in the secretory labyrinth of the type III acini of the glands. Degeneration of the salivary glands is caused by TSGDF, which is probably an ecdysteroid (Harris and Kaufman, 1981; Kaufman, 1991).

The stimulus for release of TSGDF is not yet known, but some evidence suggests that it may be stretch of the abdominal wall during feeding. Degeneration is seen only in ticks feeding past the critical weight (about 300 mg for *A. hebraeum* ; Harris and Kaufman, 1984). Neural control is implicated in the release of TSGDF, since severing the lateral and medial opisthosomal nerves, (which innervate the lateral and medial dorsoventral muscles), inhibits salivary gland degeneration (Harris and Kaufman, 1984). Since these nerves terminate in somatic muscle bundles, it is possible that stretch receptors within the muscles mediate the critical weight dependant degeneration. Release of moulting hormone (ecdysone) in *Rhodnius* (Wigglesworth, 1956) and in *Oncopeltus fasciaticus* (Nijhout, 1979, 1981) is also believed to be triggered by abdominal stretch.

In this study, ticks were artificially raised to body weights (and volumes) above the critical weight by infusion with a number of liquid media, to simulate the degree of stretch seen in ticks feeding to above the critical weight. The degree of degeneration was estimated by assaying the hemolymph for ecdysteroids, and the salivary glands for fluid secretory competence. Functional degeneration in each case could therefore be compared directly to hemolymph titer of ecdysteroids. If stretch is the stimulus for release of TSGDF, it was expected that ecdysteroids would be released and salivary gland degeneration would occur in ticks infused to weights above the critical weight.

Materials and Methods

Ticks

Amblyomma hebraeum Koch ticks from a laboratory colony were maintained at 26°C, 95% RH, and in darkness. Male and female ticks were fed on rabbits, and the females were removed at fed weights well below (50 - 150 mg) and just below (150 - 250 mg) the critical weight for salivary gland degeneration. These ticks will be referred to as 'small partially fed' (50 - 150 mg) and 'large partially fed' ticks (150 - 250 mg). Ticks above the critical weight were also removed, for use as controls, at weights of 350 - 500 mg. These ticks will be referred to as 'above critical weight' ticks.

Drugs and Media

Dissections were done with the tick immersed in a modified Hank's saline solution. Isolated glands for the secretory competence assay were held in modified TC medium 199 ('TC199', Gibco), as described below (see Appendix A for detailed composition of media).

Dopamine was prepared as a 1mM stock solution, from dopamine-HCl (Sigma), in distilled water. The dopamine stock solution was diluted to the final concentration (10 µM) with TC199. This concentration produces maximal rates of secretion in *A. hebraeum* (Lindsay and Kaufman, 1986).

Bovine serum albumin (BSA, Sigma, RIA grade) was added to TC199 at a concentration of 2 mg/mL for one of the infusion experiments. This concentration was similar to the protein concentration of hemolymph (1.97 +/- 0.03 mg/mL, mean +/- SE, as measured by the Bradford protein assay (Bradford, 1976)).

For the ecdysteroid radioimmunoassay (RIA), described below, BSA (5 mg/mL) was dissolved in 0.2 M borate buffer, pH 8.5 (referred to below as borate + BSA buffer). Radiolabelled ecdysone was purchased from New England Nuclear (ecdysone, alpha-[23,24-^{3}H(N)]-, 89.0 Ci/mmol). Ecdysone antibody (directed against E-22-

succinylthyroglobulin), was a gift from Dr. L.I. Gilbert (Biology Department, University of North Carolina, Chapel Hill).

Protein A, (5%) in borate + BSA buffer, used to separate bound from unbound ligand, was prepared by Dr. Jim Campbell (Microbiology Department, University of Alberta, Edmonton, Alta.) as described by Kessler (1981), or purchased from Sigma as a crude cell suspension.

Fluid Secretory Competence Assay

Fluid secretory competence was measured using the technique described by Harris and Kaufman (1984). Briefly, salivary glands were dissected out by removing the dorsum of the tick and carefully removing large tracheae surrounding the glands, before cutting the ducts near the site of entry to the pharynx. The glands were then transferred to TC199, and the ducts ligated with a strand of fine silk thread. Glands were then incubated at room temperature in agitated TC199 containing 10μM dopamine, an agonist of salivary fluid secretion (Kaufman, 1976), for 15 minutes. The net weight increase of the salivary glands during this time was used as an indicator of fluid secretory competence.

Ecdysteroid RIA

An RIA assay, described by Kaufman (1990), was used to monitor ecdysteroid levels in the hemolymph, ovary and eggs. Briefly, after chilling ticks on ice for 10 - 15 min., to help limit rupture of the gut, a small incision was made in the side of the tick with a razor blade scalpel. Hemolymph was collected from the wound with a 10 μL micropipette and the precise volume estimated from the length of the fluid column. In the diapause experiments (Chapter 4), ovaries and eggs were collected, gently blotted, and weighed on a Sartorius microbalance to the nearest 10 μg. Following this, the samples were homogenized in 50μL of 100% methanol. The homogenate was transferred to a

microcentrifuge tube, and the homogenizer rinsed with 50 µL aliquots of methanol until the rinse was clear. All washings were pooled together with the original homogenate.

Samples were kept in 100% methanol at -10°C, until evaporated under vacuum for further storage prior to conducting the RIA. Samples or ^{3}H-20-hydroxyecdysone (20-OHE) standards (75 µL) were incubated with 45 µL of ecdysone antibody and 90 µL of radiolabelled ecdysone, such that the total binding (B_0) was about 30% and total radioactivity per tube was about 8000 cpm. All samples were incubated for 30 minutes to overnight at 4 °C. Antibody bound ligand and free ligand were separated by addition of 20 µL of 5% protein A and centrifuged after a 5 - 15 minute incubation period. The supernatant was then aspirated off and the pellet resuspended in 50 µL of distilled water. After transferring the samples to minivials (Fisher Scientific) with 5 mL of Scintiverse E (Fisher Scientific), samples were counted on an LKB liquid scintilation counter (Model 1211 or 1217).

Abdominal Stretch by Liquid Infusion

To test the effect of artificial stretch of the abdomen on loss of fluid secretory competence and increase of hemolymph ecdysteroids, large partially-fed ticks were infused on a Harvard microlitre syringe pump for 24 hours at 13.2 +/- 0.3 µL/hr with various liquid media. To ensure constant flow, the pump was run for at least 15 minutes prior to an experiment. Large partially fed ticks were infused above the critical weight (>300 mg) to simulate the stretch seen in normally fed, above critical weight ticks. These ticks are referred to as 'large infused ticks' (LINF).

A number of controls were used in this experiment. The first, a control for the infusion process, used small partially-fed ticks infused to just under the critical weight with a volume of liquid medium proportional to that used for LINF ticks. These ticks will be referred to as 'small infused ticks' (SINF). As additional controls for the normal process of degeneration, normally-fed ticks were removed from the host at weights

equivalent to the initial and infused weights of the experimental ticks (approximately 100, 200, 400 mg). These control ticks will be referred to as small (SN), large (LN) and above critical weight (AN) normally fed ticks, respectively, in this study.

The fluid secretory competence and hemolymph ecdysteroid assays were done either immediately after infusion (Day 0) or five days after infusion (Day 5). Ticks were kept at 26°C and relative humidity (RH)>95% until tested. Ticks were examined on Day 5 in this experiment, because glands of normal ticks above the critical weight would be degenerated by this time.

Four media were used as infusates: TC199, TC199 + BSA, liquid paraffin, and nitrogen gas. Initially sterile TC199 was used as an infusate since, as we prepare it, it is isosmotic to tick tissues and ionically similar to tick hemolymph, at least for the major ion species (Na, K, Cl, Ca, Mg). Since TC199 lacks protein, in some experiments the ticks were infused with TC199 containing 2 mg/mL BSA to provide some colloid osmotic pressure . Both TC199 and TC199 with BSA may have caused dilution of some components of the hemolymph. Therefore mineral oil, which is not miscible with hemolymph, was also used as an infusate in some experiments. Because lipid soluble compounds might partition into the oil, nitrogen gas was used in the final set of inert medium experiments (see Chapter 3).

In all the above liquid infusion experiments, a single rate of infusion was used (approximately 13 µL/hr). To test if the rate of infusion itself was a factor in the proposed stretch response, some ticks were infused with sterile TC199 at approximately 33 µL/hr. In these experiments, the infusion time varied from 12 -16 hours for small partially-fed ticks, and 22 - 24 hours for large partially-fed ticks. All of these infused ticks were assayed for secretory competence, and hemolymph ecdysteroid levels.

Hemolymph Ecdysteroids during Feeding

In the course of early experiments, SN day 0 control ticks showed a noticeable ecdysteroid peak, compared to larger day 0 ticks (Fig. 2.2); thus, a series of ticks from 50 - 500 mg were assayed on day 0 for hemolymph ecdysteroid levels. Hemolymph was collected within one hour of removing the ticks from the host and assayed as already described.

Statistics

Results are reported as mean and SEM (n). Unless otherwise indicated, statistical significance was calculated using a one-way analysis of variance (ANOVA) with a multiple comparisons test. Wilcoxon's signed rank test, a nonparametric test, was used on some of the ecdysteroid data, as the data did not meet the normal distribution assumptions of an ANOVA. All statistics were done with Statview 512 (Abacus Concepts) on a Macintosh SE microcomputer and with the MIDAS statistical package of the university mainframe computer.

Results

Normally-fed Control Ticks

In normally fed ticks, secretory competence decreased after five days, post-removal from the host (Fig. 2.1). In all control groups, the decrease was significant (ANOVA, $p<0.01$), but only the AN controls had complete loss of function (a 99% reduction in rate of secretion). The secretory ability in SN and LN controls also decreased, with 35% and 76% reductions in secretion rate respectively by day 5.

Hemolymph ecdysteroid levels increased significantly only in AN ticks (Fig. 2.2), rising to five times the day 0 ecdysteroid level. Although mean ecdysteroid levels in the LN ticks appeared to increase, this was not significant (Wilcoxon's signed rank test, $p>0.05$), probably due to the high variation in the day 5 group. Similarly, hemolymph ecdysteroid levels in the SN ticks did not change significantly by day 5 (Wilcoxon's signed rank test, $p>0.05$). Also, the SN ticks had an ecdysteroid titer approximately two times that of AN ticks on day 0 (Wilcoxon's signed rank test, $p < 0.05$).

Ticks Infused with TC199

LINF ticks, infused beyond the critical weight, decreased (ANOVA, $p<0.01$) in secretory competence by 68% after five days of incubation (Fig. 2.3). SINF ticks infused with a volume of TC199 equivalent to that infused into the LINF ticks, but to weights under the critical weight, also decreased in secretory competence (about 65%) by day 5 (ANOVA, $p<0.01$, Fig. 2.4).

Hemolymph ecdysteroid levels did not increase significantly in LINF ticks infused beyond the critical weight by day 5 (Wilcoxon's signed rank test, $p>0.05$, Fig. 2.5). The SINF ticks showed an increase in ecdysteroids by day 5 (Wilcoxon's signed rank test, $p<0.01$, Fig. 2.6). Since in the above analysis, averaged data was used, any small changes in ecdysteroid titer as a function of weight would be obscured. Therefore ecdysteroid levels on day 5, when an increase in ecdysteroids was expected, were plotted for each tick

as a function of weight. At about 350 mg, in day 5 normally-fed ticks, ecdysteroid levels began to increase (Fig. 2.7). Infused ticks, however, remained at low levels of ecdysteroid (<25 ng/mL) even at infused weights of 400 or 450 mg. Stretch did not therefore induce as large an amount of hemolymph ecdysteroid increase as first appears.

TC199 + BSA Infusion

Secretory competence for LINF ticks infused with TC199 + BSA beyond the critical weight fell by 89% (ANOVA, $p<0.01$) after five days of incubation. This was not significantly different (ANOVA, $p>0.05$) from the secretory competence of AN day 5 ticks (Fig. 2.8). As with the TC199 infusions, SINF ticks infused to below the critical weight secreted 69% less fluid on day 5 than on day 0 (Fig. 2.9, ANOVA, $p<0.01$).

Hemolymph ecdysteroid concentrations in the LINF ticks infused with TC199 + BSA did not significantly increase after five days incubation (Fig. 2.10, Wilcoxon's signed rank test, $p>0.05$). The SINF ticks, also did not show a significant increase in ecdysteroid titer by day 5 (Fig. 2.11, Wilcoxon's signed rank test, $p>0.05$), and the ecdysteroid levels in infused ticks were smaller than either of the appropriate controls.

Oil Infusions

Infusion of liquid paraffin into ticks was generally toxic. Although the secretory competence of both SINF and LINF ticks fell to almost zero, the same level as AN controls, after five days of incubation (Table 2.1), the ticks did not appear healthy, having dark, fragile cuticles. Thus this method was abandoned in favor of N_2 gas infusion experiments and no further analysis was done on this data.

Table 2.1: Salivary secretory competence for SINF ticks infused with liquid paraffin over 24 hours to weights below the critical weight and LINF ticks infused to above the critical weight at day 0 and day 5 post-infusion.

	ROS (mg/15 min)	
Tick Group	Day 0	Day 5
SINF	6.56 +/- 1.25 (5)	0.67 +/- 0.23 (8)
LINF	5.17 +/- 0.94 (11)	0.05 +/- 0.05 (8)

Rapid Infusion

As shown in Table 2.2, infusion of TC199 at about 33 µL/hr with sterile TC199 caused injury in most ticks by day 5. The hemolymph and tissues of most ticks was in poor condition by day 5, with gut rupture in some ticks. After 5 days incubation, all the SINF ticks and 72% of the LINF ticks failed to secrete fluid. Also, most of these ticks showed signs of putrefaction after 5 days.

This is further illustrated in Fig. 2.12 and 2.13, which show fluid secretory competence after 5 days incubation for surviving ticks. For both LINF and SINF ticks, secretion levels fell to 0 by day 5. Day 0 secretion rates were similar to levels seen for LN and SN day 0 ticks, respectively (ANOVA, $p>0.05$).

Among the ticks surviving infusion, both LINF and SINF ticks (Fig. 2.14 and 2.15) showed no increase in 20-OHE 5 days post-infusion (Wilcoxon's signed rank test, $p>0.05$). Thus stretch at a faster rate did not stimulate release of ecdysteroids, but, since most of the ticks were in poor condition in this experiment, the physiological significance of these ecdysteroid levels are suspect.

Table 2.2: Condition of ticks infused with sterile TC199 at 33.0 +/- 0.9 µL/hr, immediately following (day 0) and five days following (day 5) infusion. Hemolymph and tissue of 'degenerated' ticks was in very poor condition.

Tick treatment	Alive	'Degenerated'
SINF day 0	8	0
SINF day 5	0	9
LINF day 0	7	0
LINF day 5	7	18

Secretion as a Function of Hemolymph Ecdysteroid Level

Since salivary gland degeneration is caused by TSGDF (probably an ecdysteroid), one would expect fluid secretory rate to be an inverse function of hemolymph ecdysteroid concentration. This was not the case, however, for a majority of the ticks in this study. As shown in Fig. 2.16 and 2.17, using the combined data of both SINF and LINF TC199 infused ticks, there was no correlation between increased ecdysteroid titer and secretory rate. Linear regression calculations gave a R^2 of 0.218 and 0.042 for Fig. 2.16 and 2.17 respectively. However, very few ticks had levels exceeding 50 ng/mL; ticks which did reach ecdysteroid titers of more than 50 ng/mL also showed decreased fluid secretory ability, but these were exceptions. Most of the ticks in this experiment had ecdysteroid titers below 20 ng/mL.

Salivary Gland Degeneration and the Critical Weight

Fluid secretory rate and ecdysteroid levels in infused ticks did not show any correlation with the previously known critical weight, unlike normally-fed ticks (Fig. 2.7). To investigate whether the amount of artificial stretch from a net increase in weight, rather than an absolute weight, was the factor controlling salivary gland degeneration, secretory competence (Fig. 2.18) and hemolymph ecdysteroid levels (Fig.

2.19) were plotted against the ratio of infused to original weight for LINF ticks. Linear regression calculations gave very low correlation coefficients (R^2 of 0.026 and 0.08 for Fig. 2.18a and b respectively). Fig. 2.19 had too few points to attempt linear regression.

In both TC199 and TC199 + BSA infusion (Fig. 2.18), secretory rates did not change in a consistent manner over the entire range of weight ratios, instead of decreasing, as expected, at weight ratios above the ratio of 2 (roughly equivalent to a weight increase beyond the critical weight). The hemolymph ecdysteroid levels for TC199 and TC199 + BSA infused day 5 ticks also did not show the expected increase with an increase in weight ratio (Fig. 2.19), although the data is, admittedly, very limited.

Hemolymph Ecdysteroids during Feeding

Since the SN day 0 control ticks showed a higher level of hemolymph ecdysteroid than the larger ticks, a series of ticks from 50 to over 400 mg were assayed for hemolymph ecdysteroids to examine this peak in greater detail. As seen in Fig. 2.20, ticks up to 150 mg had ecdysteroid levels twice that of ticks above 150 mg (ANOVA, $p<0.05$, data pooled as under and above 150 mg).

Discussion

TC199 and TC199 + BSA infusions had no specific effect on secretory rate or ecdysteroid levels in the large partially-fed ticks. Fluid secretory competence of LINF ticks infused with TC199 did not fall to the same level as AN ticks by day 5 (Fig. 2.3). TC199 + BSA infusion produced a larger decrease in the rate of secretion, approaching levels near that of AN ticks, but there was no corresponding increase in ecdysteroid titer to levels seen in the AN ticks (Figs. 2.8 and 2.10). Also neither secretory rate nor ecdysteroid titer were found to be correlated with a net increase in body weight past the critical weight with either TC199 or TC199 + BSA treatments (Fig. 2.18 and 2.19). This failure to stimulate degeneration may be due to problems in infusing the large ticks to higher weights, as will be discussed below.

Fluid secretory competence was not correlated with ecdysteroid levels in the TC199 and TC199 + BSA experiments. However, secretory rates on day 5 were generally lower than rates on day 0, and associated with slightly higher ecdysteroid concentrations (Fig. 2.16 and 2.17). Moreover, the ecdysteroid levels reached were still probably below the threshold for true salivary gland degeneration (about 30 ng/ml hemolymph, Harris and Kaufman, 1985), and they were not at levels seen for AN ticks on day 5 (approximately 50 ng/mL hemolymph, Fig. 2.2). Therefore the results of these experiments did not meet the criteria for establishing that true degeneration had occurred.

The SINF ticks showed a 65% and 69% decrease in secretory competence in TC199 and TC199 + BSA-infused ticks respectively (Fig. 2.4 and 2.9), but only the TC199-infused ticks showed an increase in ecdysteroid titers to the level of LN ticks (Fig. 2.6 and 2.11). Thus infusion of the smaller ticks did not conclusively produce the expected ecdysteroid titers and secretory competence of LN ticks. The decrease in secretory rate in both TC199 and TC199 +BSA infused LINF ticks appears to be a nonspecific effect, because there was a similar decrease in the SINF controls.

Ticks infused with sterile TC199 at about 33 µL/hr (Fig. 2.12-2.15) suffered from general necrosis of most tissues after five days incubation. The poor condition of the tick tissues plus the dark color and foul smell of the hemolymph suggests bacterial contamination may have been the cause of the internal damage, rather than effects of ecdysteroids. Since the sterile infusion medium was from several different batches, and had worked in other experiments conducted in our laboratory at that time, the deterioration was probably not due to contamination of the medium. Thus, these results were too inconclusive to establish whether salivary gland degeneration is dependent on infusion rate.

Several of the results suggest that either the method used to induce stretch or the amount of stretch produced were not sufficient for degeneration to occur. Infusion of SINF ticks with TC199 produced secretory rates and ecdysteroid increases similar to those of normally-fed ticks at weights equivalent to the infused weight (Fig. 2.4, 2.6, 2.9, and 2.11). Thus, infusion-induced stretch can produce a response expected of a heavier tick. It was difficult to infuse the large ticks very much higher than the critical weight, however, and so the infusion may not have been sufficient to cause salivary gland degeneration in the LINF ticks. Ticks infused at a faster rate reached weights well above the critical weight during infusion, but most died in incubation. Thus, data could not be collected for ticks at higher infused weights.

The SINF ticks had ecdysteroid levels much lower than either of the normally fed controls on day 0 (Fig. 2.6 and 2.11), suggesting that the hemolymph of these ticks may have been diluted with infusate. The LINF and SINF ticks in both TC199 and TC199 + BSA experiments were infused with equivalent, rather than proportional volumes, because of the difficulty in infusing the LINF ticks to weights much higher than the critical weight. Thus, SINF ticks may have received a relatively larger volume of infusate than the LINF ticks did. The resulting dilution of the hemolymph may be the cause of the reduced hemolymph ecdysteroid titers seen in the SINF day 0 ticks.

Since oil and high infusion rates (33.0 µL/hr) were toxic, and since other liquid infusion could not induce degeneration, nitrogen gas infusion was also attempted, the results of which are discussed in the next chapter.

The decreased fluid secretory response in the absence of the expected increase in ecdysteroid titer in LINF ticks suggests that stretch could affect hormone release separately from the secretory response. This decrease in fluid competence is probably just the recoverable fluid loss seen in ticks under the critical weight however (Harris and Kaufman, 1984). Normally-fed control ticks, above the critical weight, showed a large increase in ecdysteroid titer after five days incubation (Fig. 2.7), correlated with a decrease in secretory rate, while the LN ticks showed only the fluid secretory competence decrease. Thus in normally-feeding ticks, release of ecdysteroids and salivary gland function are linked to the same critical weight. Infusion with a liquid medium, it seems, was not capable of stimulating increased titers of the hormone in the hemolymph, while the decreased secretory response normally seen in ticks under the critical weight was stimulated. This may be because the large volume of injected fluid diluted the hemolymph, such that any increase in ecdysteroid release was masked. The N_2 gas infusion experiment (Chapter 3) was done with hopes of avoiding the interaction of dilution effects of liquid media.

Although salivary gland degeneration could not be induced by infusion of liquid into ticks, other interesting observations were seen in the control animals. In the normally-fed ticks, SN and LN ticks had no statistically significant change in ecdysteroid concentration by Day 5 (Fig. 2.2), but the SN ticks showed a trend of reduced ecdysteroids. AN ticks had increased ecdysteroid levels within the range of concentration known to induce degeneration (30-100 ng/mL hemolymph, Harris and Kaufman, 1985), and data for LN ticks also suggested an ecdysteroid increase. This suggests some controlling mechanism may exist that is linked to a weight-monitoring system allowing

small partially-fed ticks to maintain low titers of ecdysteroids in the hemolymph when the tick is off the host.

The ecdysteroid levels in SN ticks at day 0 were also interesting results (Fig. 2.2). The higher levels of ecdysteroids (about 20 ng/mL) in these ticks, and the decrease in ecdysteroids to about 10 ng/mL in ticks larger than 150 mg (Fig. 2.20) may be related to development of the cuticle to allow for expansion of the abdomen while feeding. In *A. hebraeum* synthesis of endocuticle occurs during the slow stage of feeding, when tick body weights are increasing from 50 to 500 mg. The most rapid increase in dry weight of cuticle is seen at about 100 to 150 mg (Connat, et al, 1985). This growth phase could be due to activation of the cuticle-secreting epidermal cells by ecdysteroids, in preparation for deposition of new cuticle, as occurs in insects (Wigglesworth, 1963). In nymphal and adult argasid ticks, a prepulse of ecdsyone is correlated with mitosis in the hypodermal cells of the epidermis, in preparation for moulting (Solomon et al, 1982). Ixodid ticks also have a mitotic phase in the epidermal cells for production of extensible cuticle (Lees, 1952). This seems to provide further evidence for the hypothesis of regulation of hemolymph ecdysteroid titers by stretch, in addition to that of the ecdysteroid increase associated with degeneration in large ticks, since the peak in ecdysteroid titer correlated to the period of cuticle growth seems to be linked to a particular range of body weight.

In summary, liquid infusion as a method of inducing abdominal stretch failed to stimulate salivary gland degeneration according to the criteria established for this study. In the next chapter, results of the air infusion experiment, designed to avoid the problem of dilution of the hemolymph, are discussed.

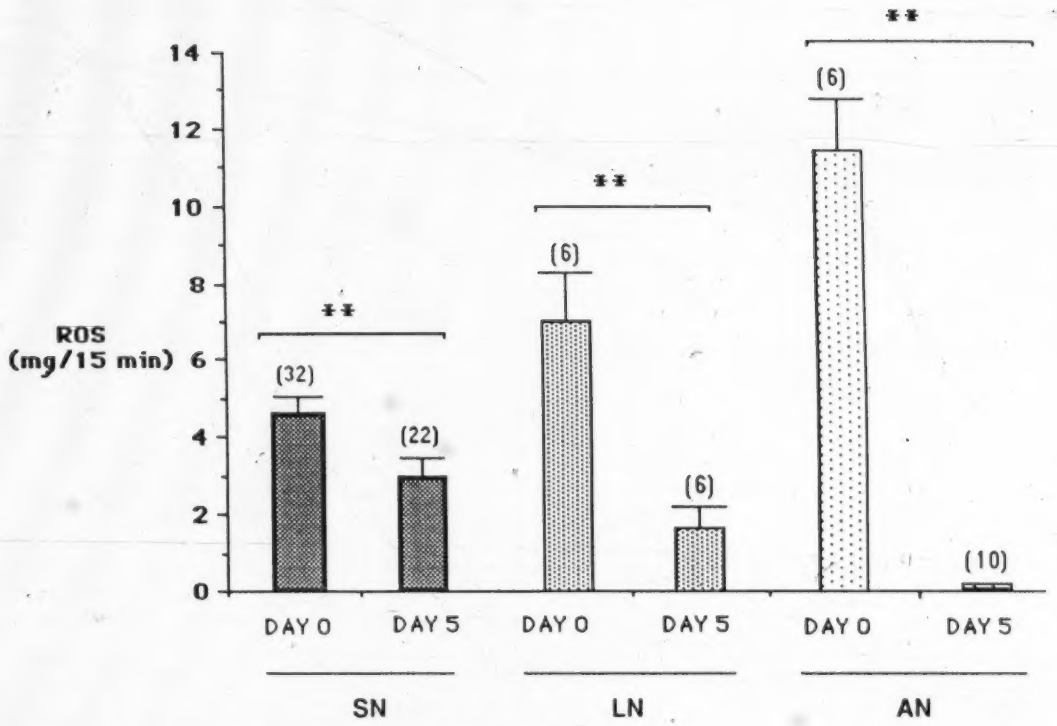

Fig. 2.1: The rate of fluid secretion (ROS) of normally-fed control *A. hebraeum* ticks immediately after (day 0) and five days after (day 5) removal from the host. Significant differences between days are indicated with asterisks (ANOVA, **: $p < 0.01$). Standard error and sample size are shown. Abbreviations as described in text.

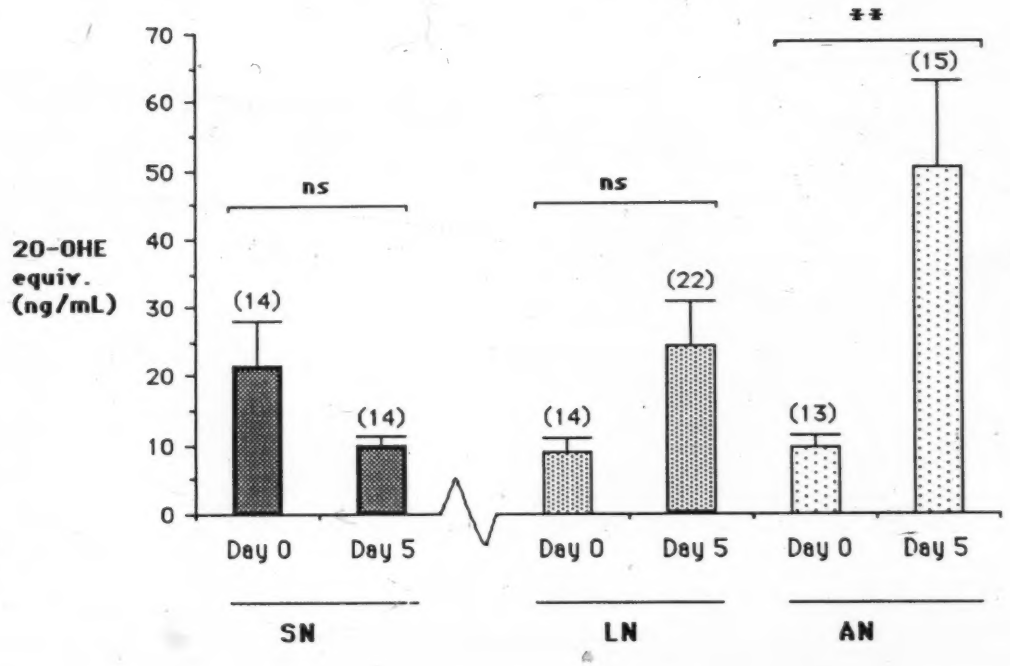

Fig. 2.2: Hemolymph 20-OHE concentrations in normally-fed control *A. hebraeum* ticks immediately after (day 0) and five days after removal from the host (day 5). Standard error and sample size are shown. Significance is indicated with asterisks (Wilcoxon's signed rank test, **: $p < 0.01$, ns: $p > 0.05$). Abbreviations as described in text.

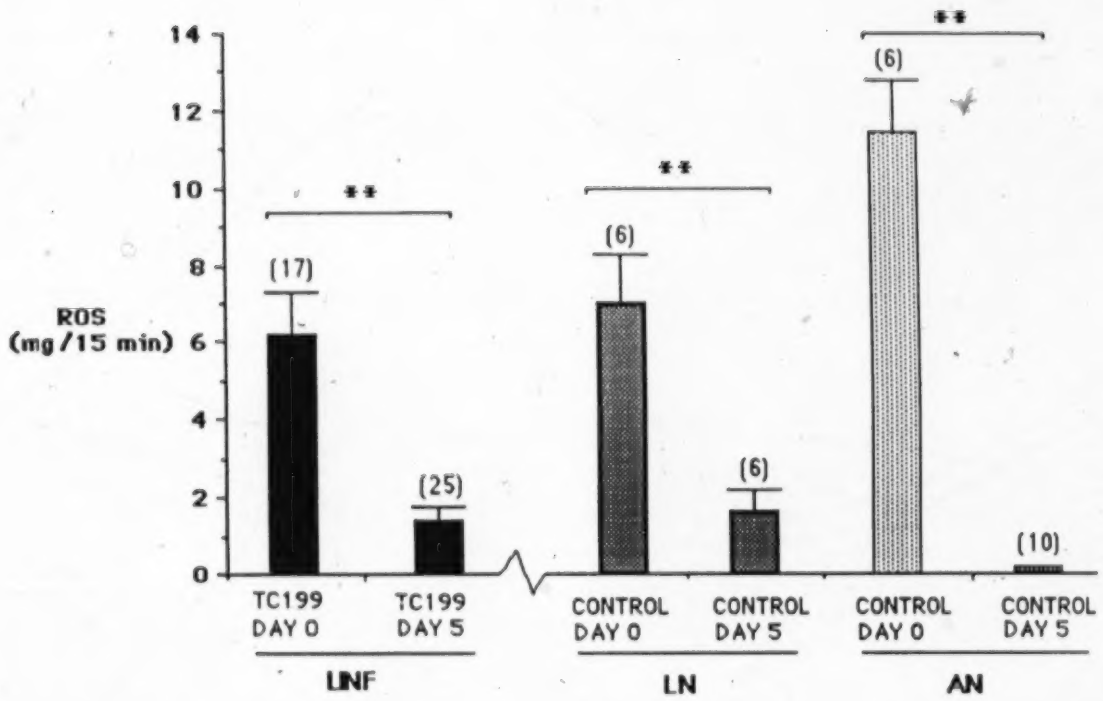

Fig. 2.3: The rate of fluid secretion (ROS) of LINF *A. hebraeum* ticks infused beyond the critical weight with TC199, and normally-fed controls, immediately after (day 0) and five days after infusion or removal from the host (day 5). Significant difference is indicated with asterisks (ANOVA, **: $p < 0.01$). Standard error and sample size are shown. Abbreviations are as described in text. Data for LN and AN ticks are taken from Fig. 2.1.

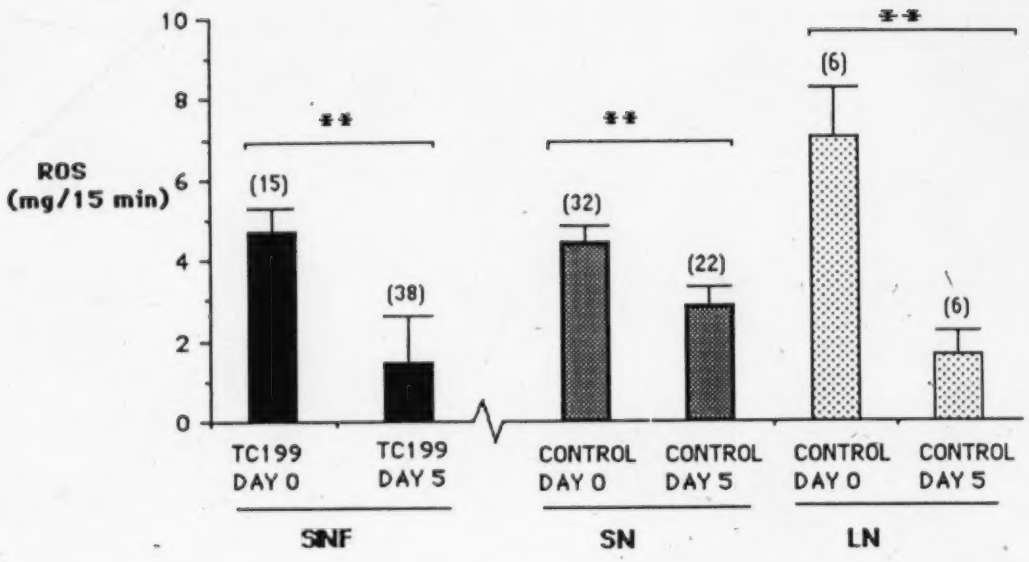

Fig. 2.4: The rate of fluid secretion (ROS) of SINF *A. hebraeum* ticks infused to under the critical weight with TC199. Sample size and standard error are shown. Significant differences between days are indicated with asterisks (ANOVA, **: $p < 0.01$). Abbreviations are as outlined in text. Data for SN and LN ticks are taken from Fig. 2.1.

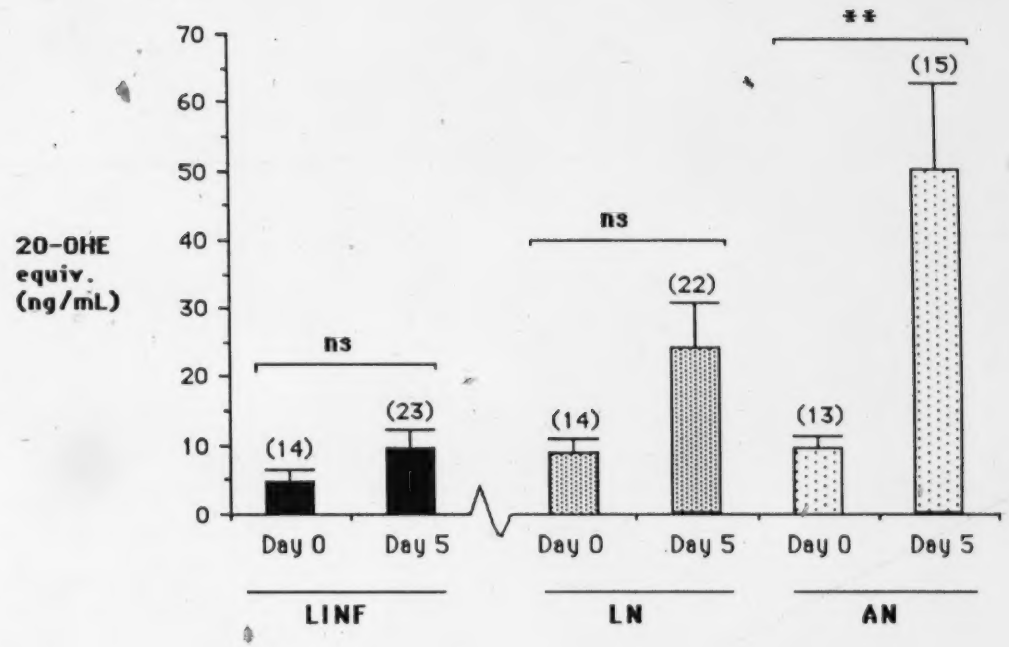

Fig. 2.5: The hemolymph 20-OHE titers in LINF *A. hebraeum* ticks infused beyond the critical weight with TC199 and normally-fed control ticks immediately after infusion, or removal from the host (day 0) and after five days incubation (day 5). Significant difference between days is shown with asterisks (Wilcoxon's signed rank test, **: $p<0.01$, **ns**: $p>0.05$). Abbreviations are as described in text. Data for LN and AN ticks are taken from Fig. 2.2.

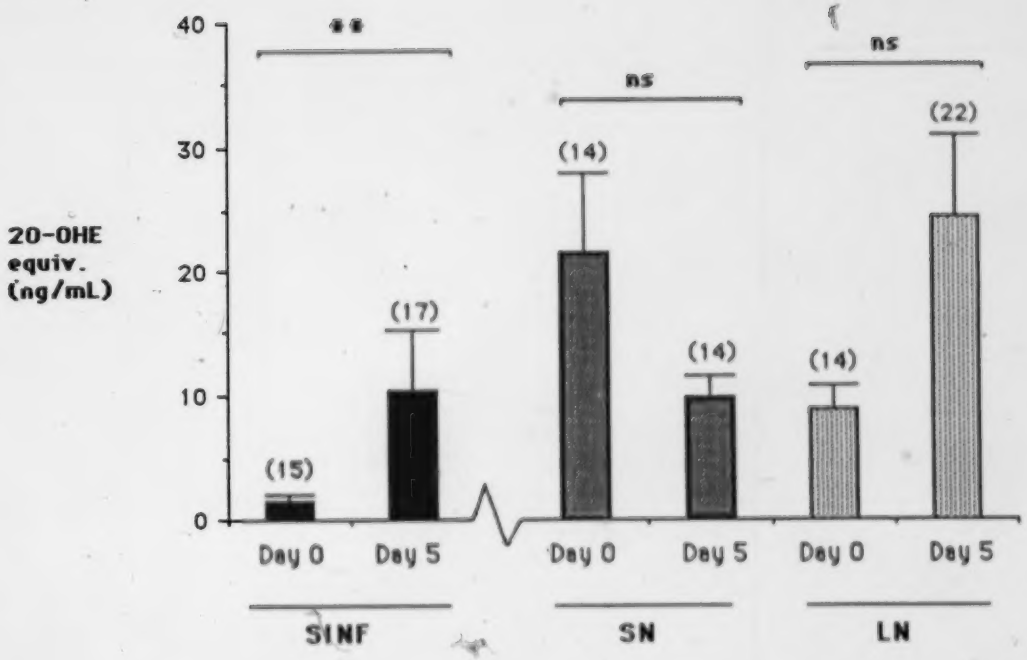

Fig. 2.6: Hemolymph 20-OHE titers in SINF *A.hebraeum* ticks infused to weights below the critical weight with TC199 and normally-fed control ticks immediately after infusion, or removal from the host (day 0) and five days after incubation (day 5). Significant differences between days are indicated with asterisks (Wilcoxon's signed rank test, **: $p < 0.01$, **ns**: $p > 0.05$). Standard error and sample size are shown. Abbreviations are as shown in text. Data for SN and LN ticks are taken from Fig. 2.2.

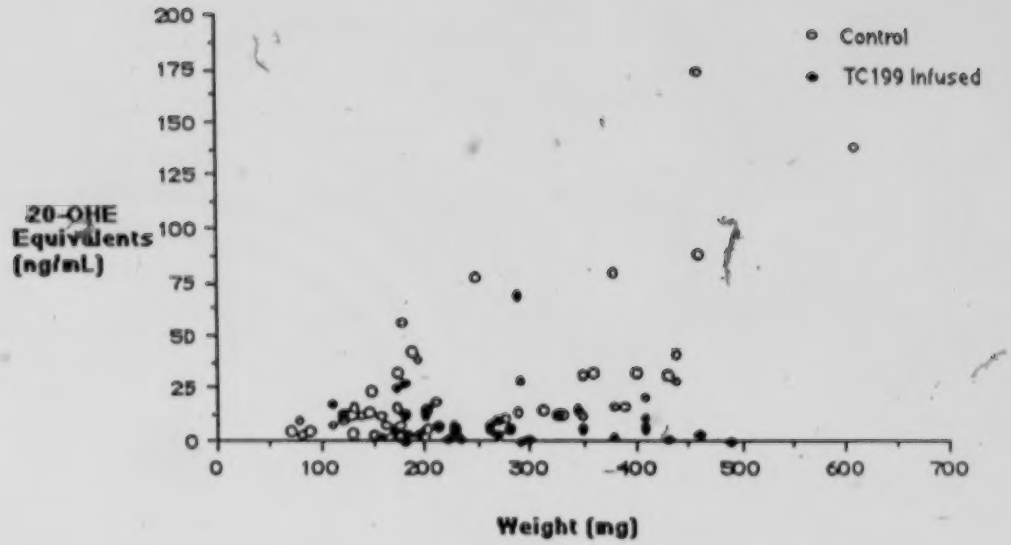

Fig. 2.7: Hemolymph ecdysteroid concentration in normally-fed controls (◦) and TC199 infused (•) *A. hebraeum* ticks after five days incubation as a function of weight after infusion or removal from the host. Data are replotted from Figs. 2.2, 2.5 and 2.6.

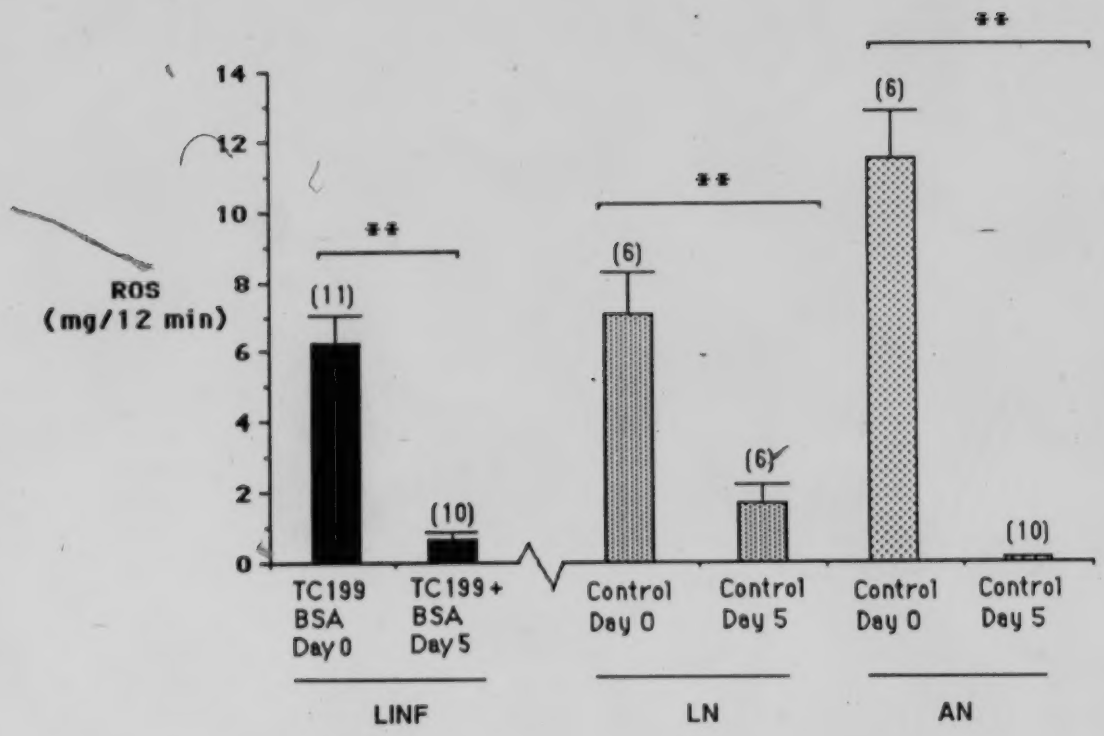

Fig. 2.8: The rate of fluid secretion (ROS) of TC199 + BSA infused LINF ticks and normally-fed control *A. hebraeum* ticks immediately after infusion or removal from the host (day 5). Sample sizes and standard error are shown. Significant differences are shown with asterisks (ANOVA, **: $p < 0.01$). Abbreviations are as described in text. Data for LN and AN ticks are taken from Fig. 2.1.

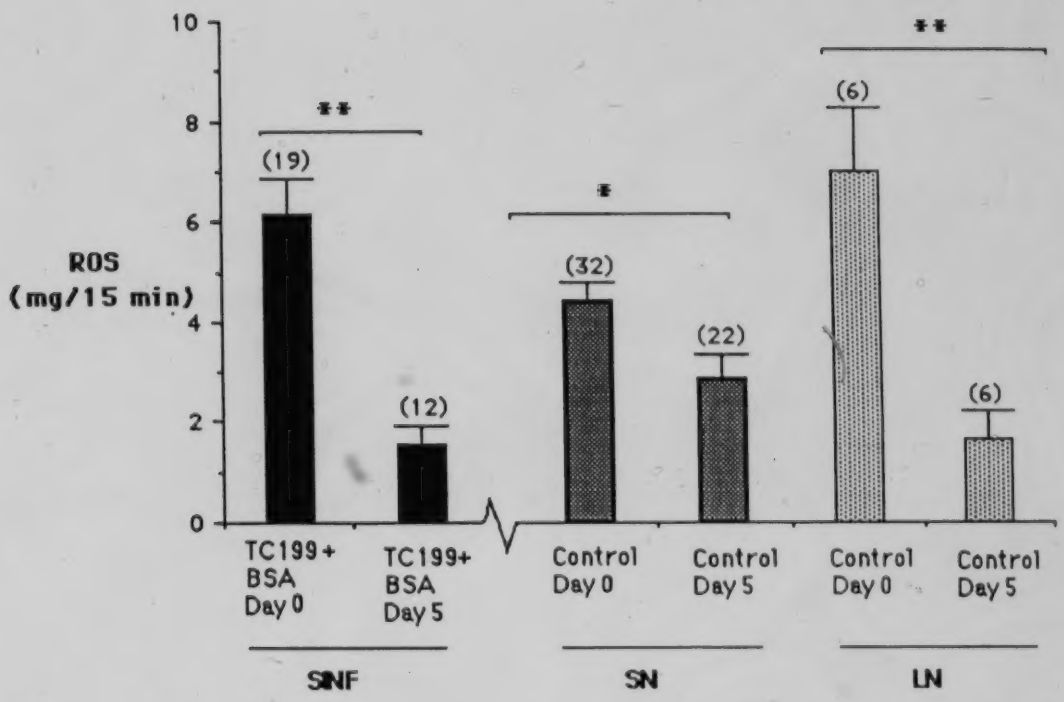

Fig. 2.9: Rate of fluid secretion (ROS) of TC199 + BSA infused SINF *A. hebraeum* ticks and control normally-fed control ticks immediately after (day 0) and five days after (day 5) infusion or removal from the host. Standard error and sample size are shown. Significant differences between days are shown with asterisks (ANOVA, *: $p < 0.05$, **: $p < 0.01$). Abbreviations are as described in text. Data for SN and LN ticks are taken from Fig. 2.1.

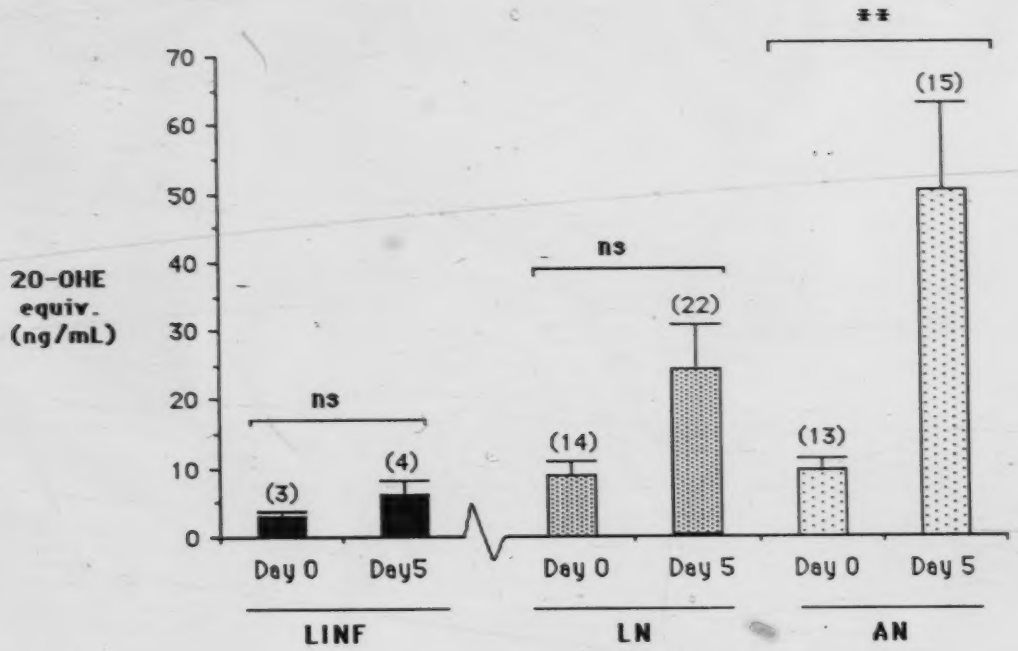

Fig. 2.10: Hemolymph 20-OHE titers in LINF *A. hebraeum* ticks infused with TC199 + BSA beyond the critical weight. Sample size and standard error are shown. Significant differences are indicated with asterisks (Wilcoxon's signed rank test, **: $p < 0.01$, ns: $p < 0.05$). Abbreviations are as described in text. Data for LN and AN ticks are taken from Fig. 2.2.

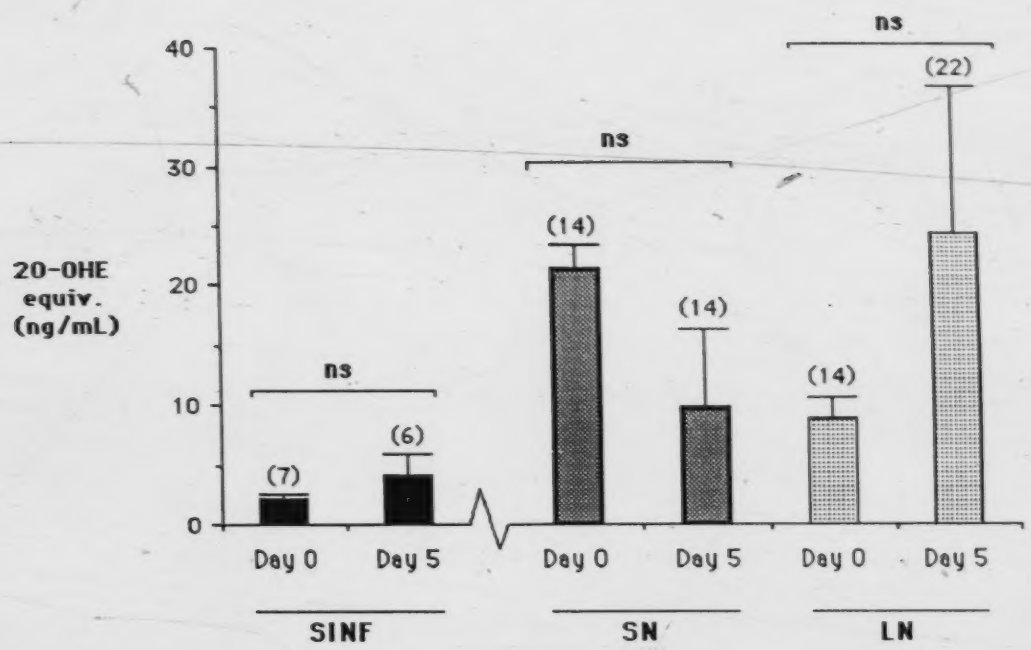

Fig. 2.11: Hemolymph 20-OHE titers in SINF *A. hebraeum* ticks infused with TC199 + BSA to weights below the critical weight. Sample size and standard error are shown. Significant differences between days are shown with asterisks (Wilcoxon's signed rank test, **ns**: $p > 0.05$). Abbreviations are as described in text. Data for SN and LN ticks are taken from Fig. 2.2.

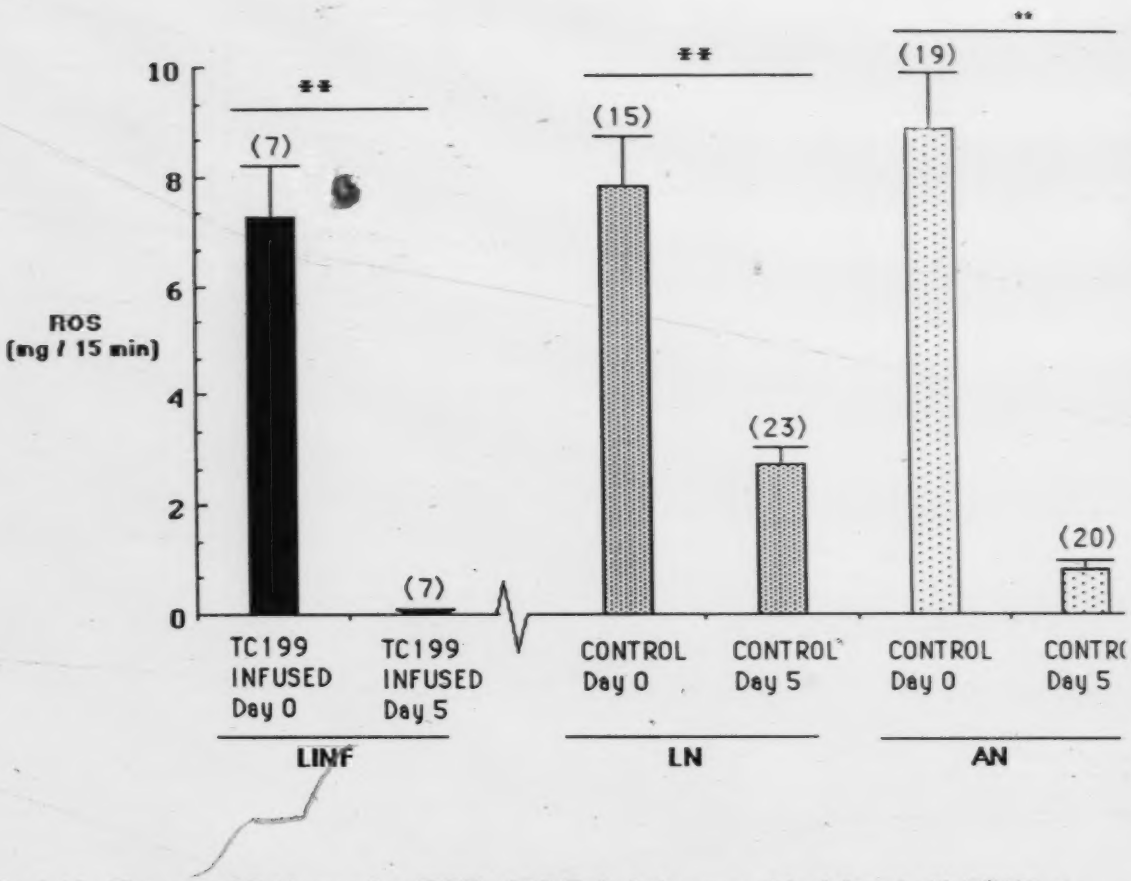

Fig. 2.12: The rate of fluid secretion (ROS) of LINF *A. hebraeum* ticks infused with TC199 at 33 +/- 0.9 µL/hr to above the critical weight, and normally-fed control ticks immediately after (day 0) and five days after infusion, or removal from the host (day 5). Significant difference is indicated with asterisks (ANOVA, **: $p < 0.01$). Standard error and sample size are shown. Abbreviations are as described. Data for LN and AN ticks includes data from Fig. 2.1 plus control data collected during this experiment.

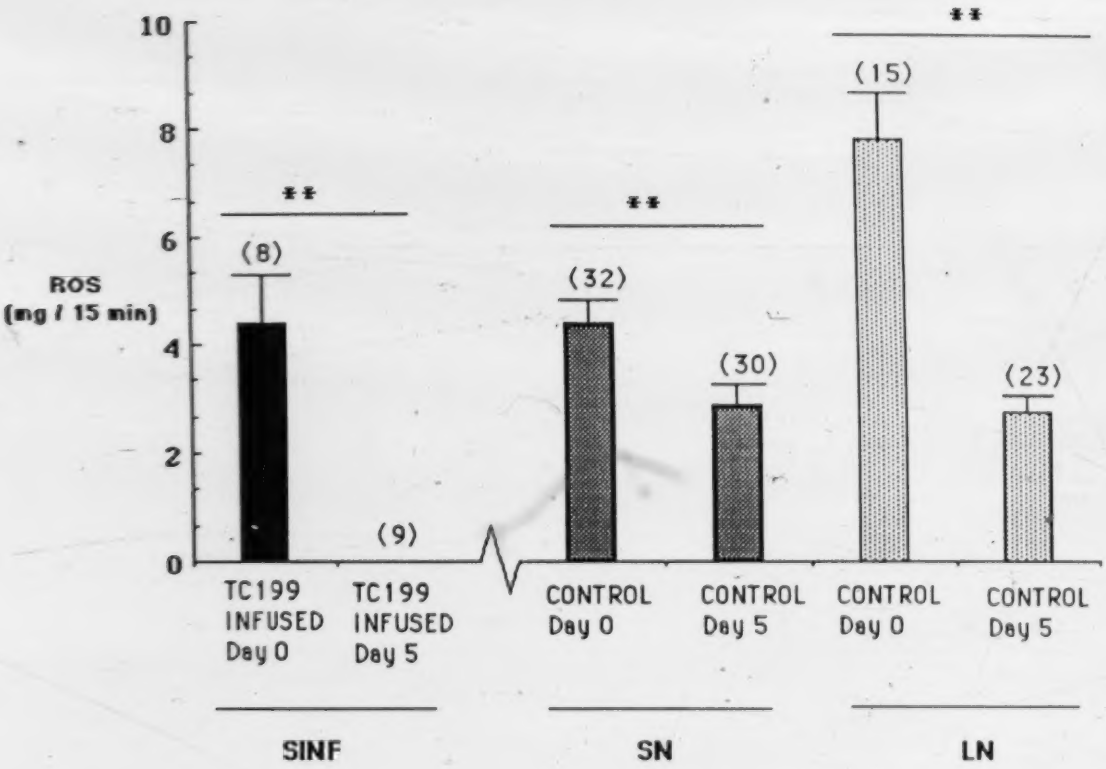

Fig. 2.13: The rate of fluid secretion (ROS) of SINF *A. hebraeum* ticks infused with TC199 at 33 +/- 0.9 µL/hr to just under the critical weight, and normally-fed control ticks immediately after (day 0) and five days after infusion, or removal from the host (day 5). Significant difference is indicated with asterisks (ANOVA, **: $p < 0.01$). Standard error and sample size are shown. Abbreviations are as described in text. Data for SN and LN ticks includes data from Fig. 2.1 and control data collected during this experiment.

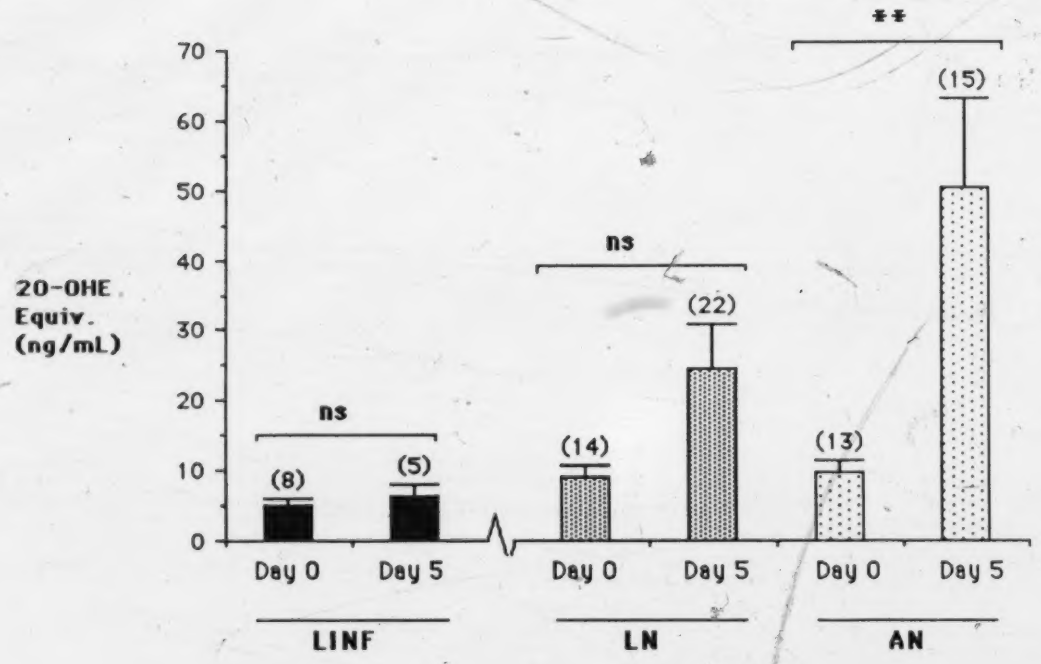

Fig. 2.14: Hemolymph ecdysteroid levels of LINF *A. hebraeum* ticks infused with TC199 at a rate of 33 +/- 0.9 µL/hr to above the critical weight and survived the treatment, and normally-fed control ticks immediately after (day 0) and five days after infusion, or removal from the host (day 5). Significant difference is indicated with asterisks (Wilcoxon's signed rank test, **: $p < 0.01$, **ns**: $p > 0.05$). Standard error and sample size are shown. Abbreviations are as described in text. Data for LN and AN ticks are taken from Fig. 2.2.

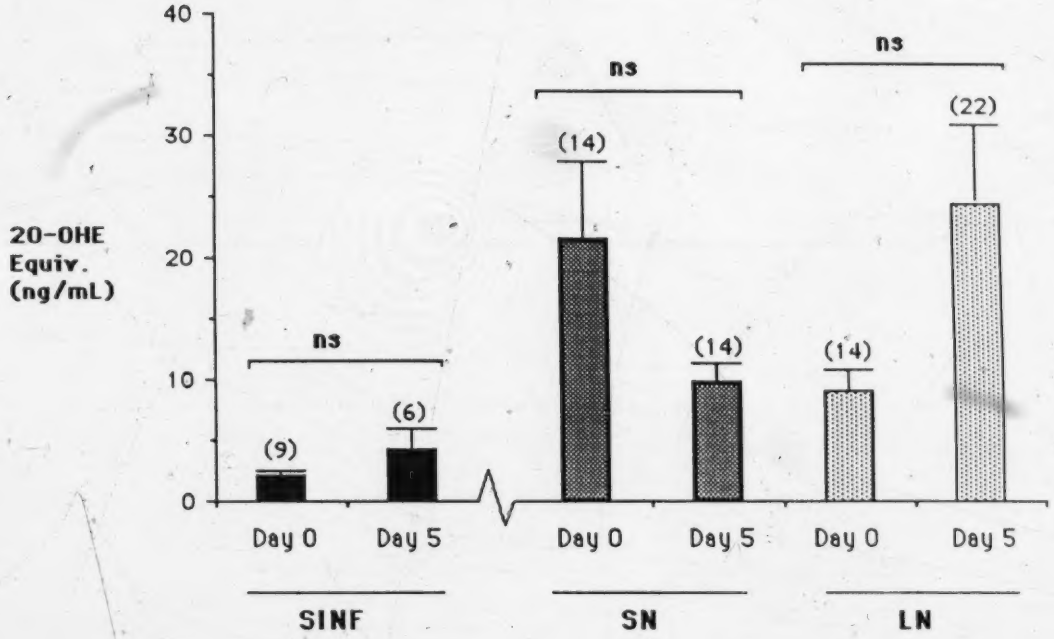

Fig. 2.15: Hemolymph ecdysteroid levels of SINF *A. hebraeum* ticks infused with TC199 at 33 +/- 0.9 µL/hr to above the critical weight and survived the treatment, and normally-fed control ticks immediately after (day 0) and five days after infusion, or removal from the host (day 5). Significance is indicated with asterisks (Wilcoxon's signed rank test, **ns**: $p > 0.05$). Standard error and sample size are shown. Abbreviations are as described in text. Data for SN and LN ticks are taken from Fig. 2.2.

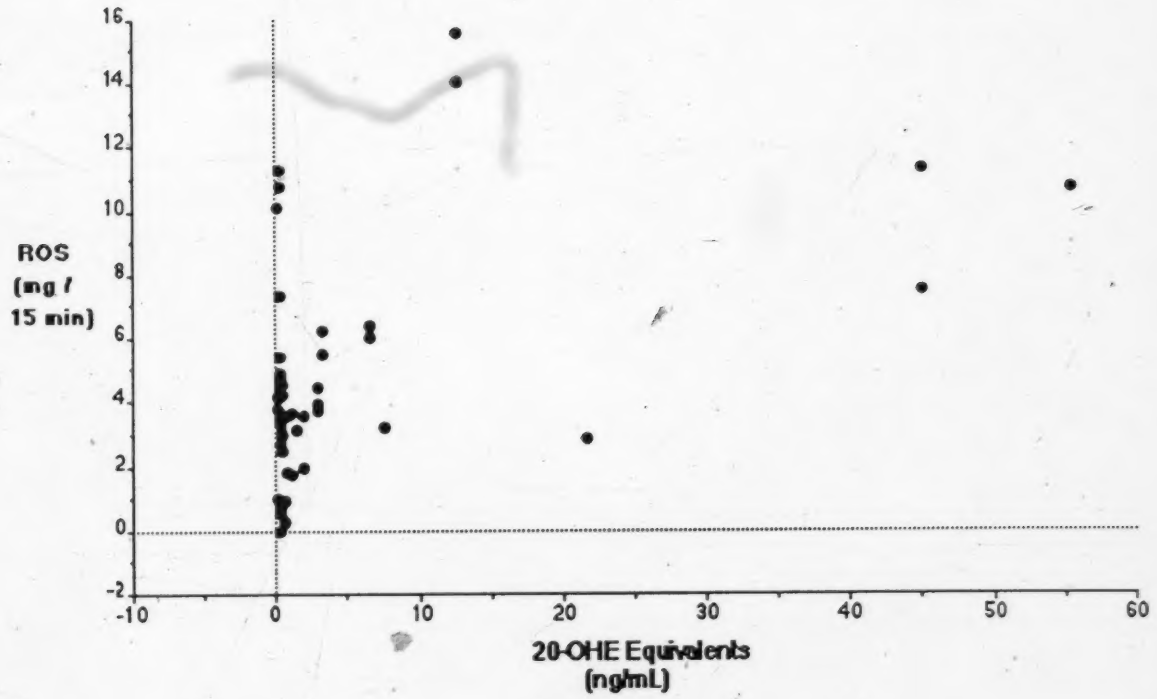

Fig. 2.16: The rate of fluid secretion (ROS) as a function of 20-OHE titer in *A. hebraeum* ticks infused with TC199 immediately after infusion (day 0). Graph represents data for SINF and LINF ticks combined, taken from Figs. 2.3, 2.4, 2.5 and 2.6. $R^2 = 0.218$.

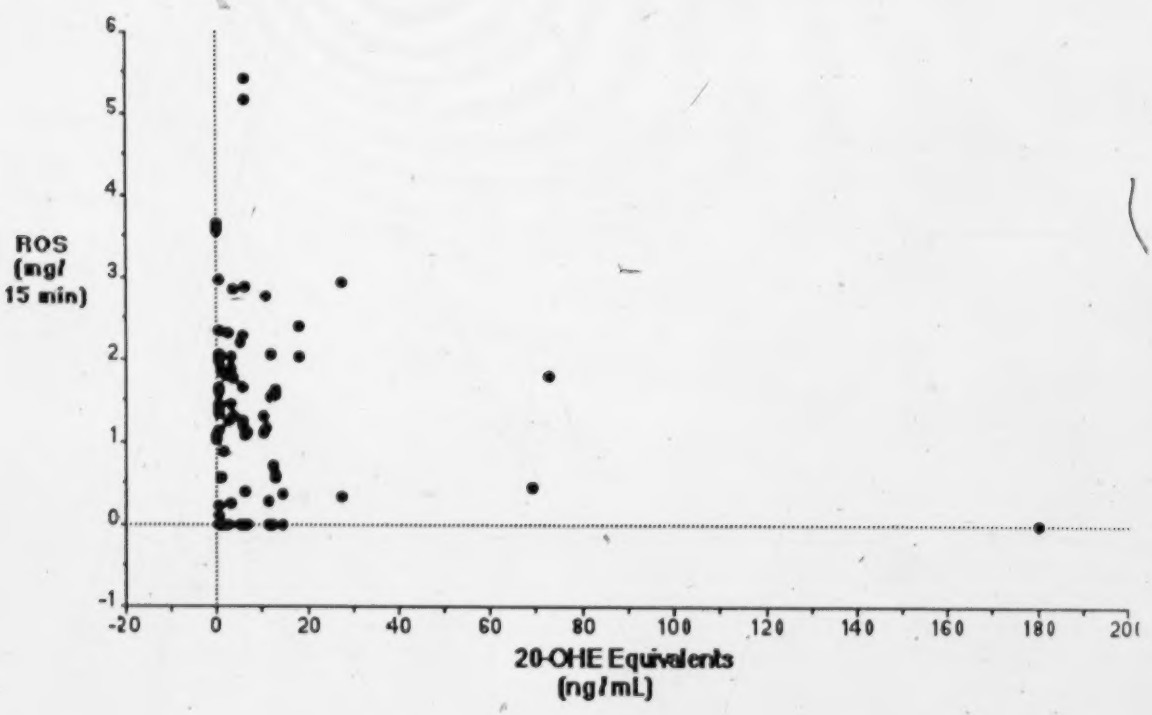

Fig. 2.17: The rate of fluid secretion (ROS) as a function of hemolymph ecdysteroid concentration in *A. hebraeum* ticks infused with TC199, after five days incubation (day 5), following infusion. Data represents combined data for SINF and LINF ticks, taken from Figs. 2.3, 2.4, 2.5 and 2.6. $R^2 = 0.042$.

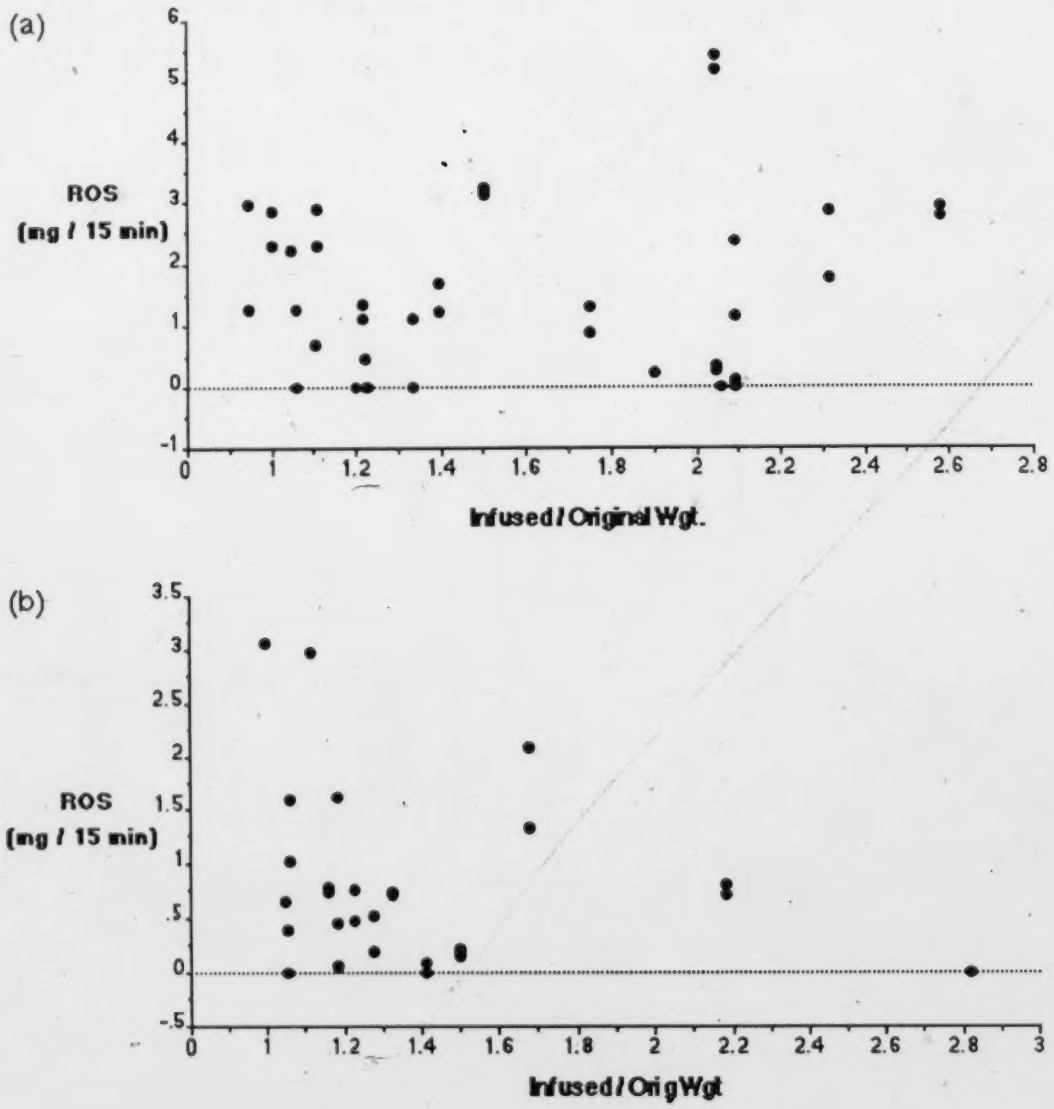

Fig. 2.18: The rate of fluid secretion (ROS) of LINF *A. hebraeum* ticks as a function of the ratio of infused to original weights in ticks infused with TC199, R^2 = 0.026 (a), or with TC199 + BSA, after five days incubation (day 5), R^2 = 0.08 (b). Data taken from Figs. 2.3 and 2.8.

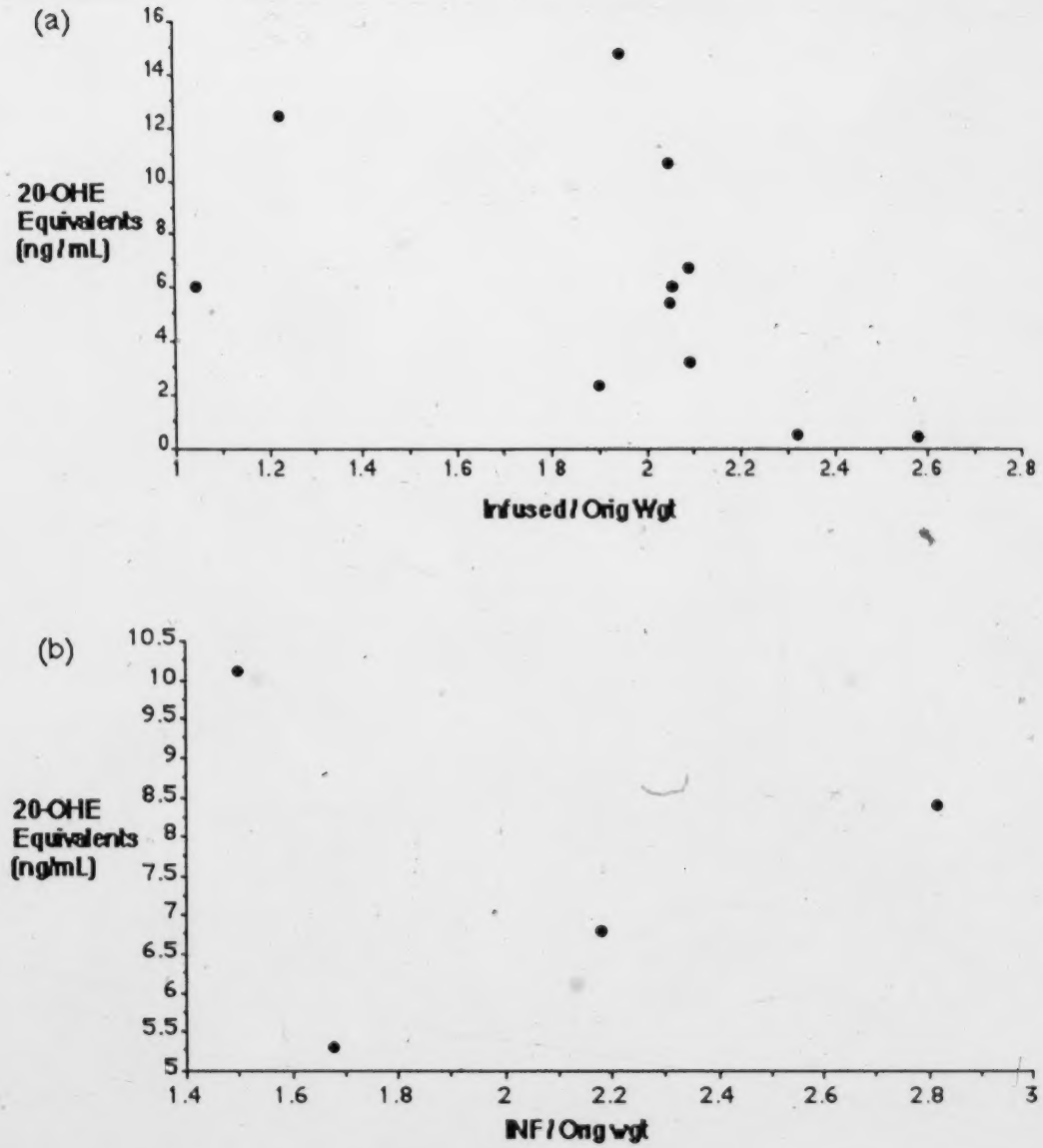

Fig. 2.19: Hemolymph ecdysteroid titers as a function of the ratio of infused to original weight ratios, for LINF *A. hebraeum* ticks, infused with TC199 (a) and TC199 + BSA (b) to beyond the critical weight, after five days of incubation (day 5), postinfusion. Data taken from Figs. 2.5 and 2.10.

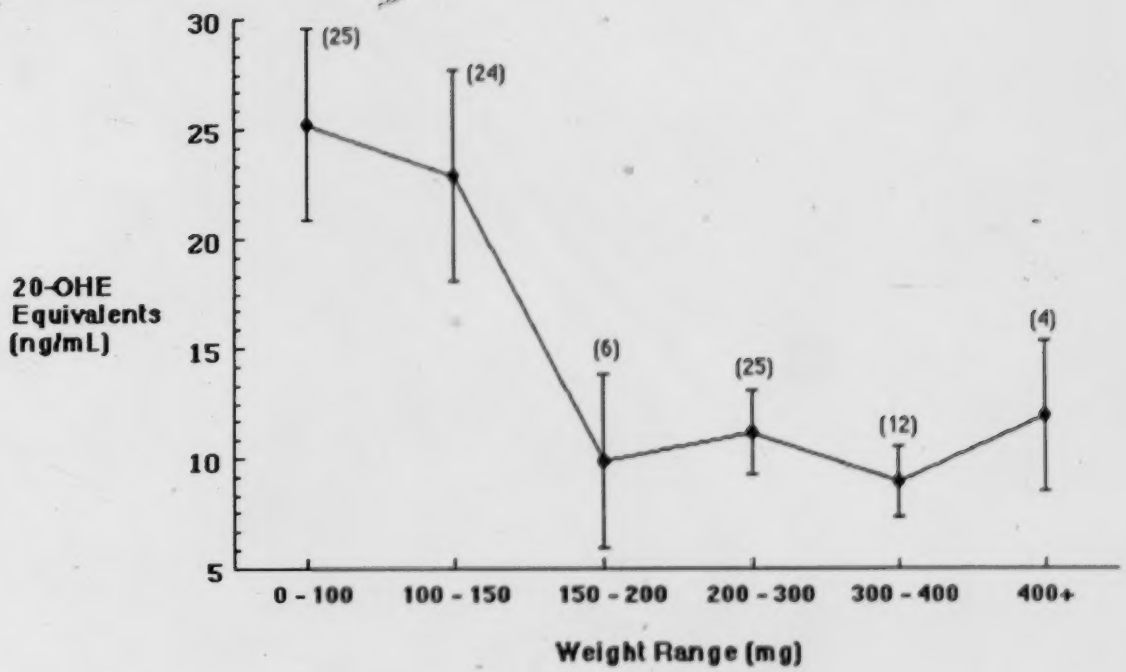

Fig. 2.20: Hemolymph ecdysteroid concentrations in normally-fed *A. hebraeum* ticks of weights from less than 50 mg to above 400 mg, on the day of removal from the host (day 0).

CHAPTER THREE

Stretch of the Abdomen by Infusion with Nitrogen Gas

Introduction

As outlined in Chapter 1, stretch of the abdomen may regulate the release of TSGDF. However, infusion of liquid into the hemocoel to simulate the abdominal stretch occurring during feeding failed to induce physiological degeneration (Chapter Two). Artificial stretch of the abdomen has been used in studies on hormone release in at least two insect species with mixed success. Nijhout (1979, 1981) induced moulting in the milkweed bug, *Oncopeltus fasciaticus*, by stretching the abdominal cuticle with saline or air injection into the hemocoel. As Nijhout had used air injection successfully, inflation of ticks with a moist inert gas (N_2) was attempted to stretch the abdomen with hope of inducing degeneration and ecdysteroid release in *A. hebraeum* .

Materials and Methods

Abdominal Stretch by Nitrogen Gas Infusion

Since inflation with nitrogen caused a volume increase without increasing weight, tick weight could not be used to estimate the relative amount of abdominal stretch. Instead, tick volume was estimated by measuring the length, height, and width of each tick with Manostat calipers prior to, and following infusion. Volume was calculated by the formula for an ellipse of rotation, which roughly approximates tick shape,

$$V = 4/3\ \pi\ abc$$

where a = 1/2 length, b=1/2 height and c= 1/2 width. Measurement of volume is highly correlated to weight in *A. americanum* (Hume, et al, 1985), and this was confirmed in *A. hebraeum* in this study (Fig. 3.1). Therefore, relative increases in volume can be used as a measure of stretch in experimental ticks.

As in the liquid infusion experiment, large partially-fed ticks, still below the critical volume, were inflated to above the 'critical volume' (the volume equivalent to the critical weight, approximately 225 mm^3, Fig. 3.1), over a 24 hour period, with 5 - 8 pounds per square inch (psi) moist N_2 gas. The tick was impaled through the scutal fold on a 30 gauge needle, and inflated in a sealed chamber, kept at 100% relative humidity at room temperature by distilled water in a bottom compartment of the chamber (Fig. 3.2). The high humidity was used to try to reduce desiccation of the ticks by the injected nitrogen. Cyanoacrylate glue ('Loctite', Loctite Corporation, Newington, CT) was used to seal the needle insertion wound before and after inflation, to prevent leakage of the gas.

Small partially-fed ticks were also inflated under the same conditions, but for shorter times, with a proportional volume increase, to volumes just under the critical volume (225 mm^3). These served as controls for the infusion process. The two sets of ticks will be referred to as large inflated ticks (LINF) and small inflated (SINF) ticks in the results and discussion section of this chapter.

Following inflation, ticks were randomly assigned to one of two incubation times: day 0, which were assayed immediately following inflation, or day 2, which were assayed after two days of incubation (26° C, 95% R.H., in darkness). An incubation time shorter than the normal degeneration period was necessary because I discovered in preliminary experiments that considerable dehydration of the tick occurred by the fifth day of incubation, despite precautions taken to water-saturate the gas and maintain the ticks at 100% relative humidity. The ticks were assayed for salivary secretory competence and hemolymph ecdysteroid titer as described in Chapter Two.

For additional controls, normally-fed ticks were removed from the host at weights equivalent to the initial and target volumes of the infused ticks (small (SN), large (LN) and above critical volume (AN) partially-fed ticks) and assayed, as the inflated ticks were, on days 0 and 2.

Results

Normally-fed control ticks

Fluid secretory competence of normally-fed control ticks (Fig. 3.3), two days after removal from the host varied with the weight of the tick. SN and AN ticks showed no significant change in secretory ability by day 2 (ANOVA,$p>0.05$), while LN ticks showed a slight but significant decrease in ROS (ANOVA, $p<0.05$).

Hemolymph ecdysteroid levels in all weights of control ticks showed no significant change in ecdysteroid level after two days incubation (ANOVA, $p>0.05$, Fig. 3.4). As seen in Chapter Two, day 0 SN controls had ecdysteroid levels of approximately two times that of AN day 0 ticks (ANOVA, $p<0.05$).

Nitrogen Infused Ticks

Secretory competence of LINF ticks (Fig. 3.5) inflated beyond the critical volume with nitrogen did not change from the secretory rate expected for LN ticks by day 2. The fluid secretory competence of day 2 LINF ticks was not significantly different from the day 2 LN controls, although it was close to being significant ($p = 0.058$). It was still significantly lower than day 2 AN controls, (ANOVA, $p< 0.05$). SINF ticks (Fig. 3.6), inflated to below the critical volume with a volume of nitrogen proportional to that used for LINF ticks, showed a highly significant decrease (ANOVA, $p<0.01$) by day 2, similar to the decrease seen in the LN controls. However the day 2 secretory level was not significantly different from either the SN or LN controls.

Hemolymph ecdysteroid levels in LINF ticks (Fig. 3.7) showed no significant change (ANOVA, $p>0.05$) by day 2, similar to both the LN and AN controls. Both the day 0 and day 2 levels were much greater than the controls however (6 times and 7 times the day 0 and day 2 LN control levels, respectively). SINF ticks (Fig. 3.8), the controls for inflation, also showed no significant change in ecdysteroid titer by day 2 (ANOVA, $p>0.05$), and both

day 0 and day 2 levels were again much greater than both the SN and LN controls (4 times and 16 times higher than SN day 0 and day 2 levels, respectively).

Volumes of hemolymph collected from these ticks were quite low, even for the day 0 ticks, compared to the controls, as shown in Table 3.1, suggesting that the nitrogen treatment dried out the ticks.

Table 3.1: Volume of hemolymph collected for ecdysteroid assay in control and nitrogen inflated ticks, on removal from inflation apparatus (Day 0), and after two days incubation (Day 2). All values expressed in mean +/- standard error. Sample size is included in brackets. Differences between the SINF and LINF ticks and their respective controls within each day 0 and day 5 group are indicated with symbols beside the significantly different controls (ANOVA, $p<0.05$, (*) significant difference from SINF ticks, (•) LINF ticks).

	Volume of Hemolymph Collected (µL)**	
Tick	Day 0	Day 2
SINF	3.3 +/- 0.71 (12)*	6.0 +/- 0.67 (10)*
LINF	8.2 +/- 1.0 (14)•	9.4 +/- 1.5 (9)•
SN	11.7 +/- 7.0 (15)*	9.2 +/- 0.8 (6)* •
LN	18.6 +/- 2.7 (13)*	12.5 +/- 1.3 (8)* •
AN	20.1 +/- 3.2 (14)* •	16.3 +/- 3.4 (12)* •

** True hemolymph volume can only be estimated with a space marker, such as inulin. These values do not reflect true hemolymph volume.

To check whether the high ecdysteroid titers seen in inflated ticks were due to stretch, and not just to the reduction of hemolymph volumes, ecdysteroid levels were plotted against the ratio of inflated to original volume, an indicator of relative stretch of the abdomen. There was no correlation for either SINF or LINF ticks (Fig. 3.9 and 3.10).

Discussion

Inflation with nitrogen gas, like liquid infusion, did not appear to induce salivary gland degeneration according to criteria established for this study. Both the secretory competence of the glands and the hemolymph ecdysteroid titers of infused ticks were not equivalent to AN ticks, and there was no correlation between hemolymph ecdysteroid levels and the infused to original volume ratio (Fig. 3.9, 3.10). All of these would be expected to occur if specific degeneration had been induced.

Nitrogen infusion appeared to dehydrate the ticks, as indicated by the high ecdysteroid titers in infused ticks, as well as the low volumes of hemolymph available for collection from infused ticks, as compared to control ticks. Salivation is known to be stimulated soon after infusion of large doses of saline into the tick (Kaufman, *et al* , 1980). The stretch induced by inflation with gas may have similarly triggered salivation. Indeed, the ticks salivated quite often after infusion; the vials they were stored in during the incubation period would often have noticeable quantities of saliva in them within the first day of incubation. Since gas inflation stretched the abdomen without adding any additional fluid to the hemolymph however, the ticks would have secreted fluid from the original hemolymph volume, and so dehydrated themselves. There may also be a component of these high ecdysteroid titers which is due to stimulation of TSGDF release, but due to the effects of salivation noted above, it is impossible to confirm this. However, the glands functioned well with respect to the secretory competence assay on both day 0 and day 2, so this did not seem to affect the general 'health' of the tick adversely. Strangely, hemolymph volumes actually seemed to increase in the infused ticks by day 2, but still not quite to the level seen in the normally-fed controls. Perhaps this represents a movement of water from the cells to the hemolymph in response to a possible increase in colloid osmotic pressure.

Although nitrogen infusion did not appear to induce salivary gland degeneration, an interesting observation arose from the day 2 control data, especially when compared to

trend to increasing titers (Fig. 2.2), but, by day 2, both LN and AN ticks showed no increase in ecdysteroid titer (Fig. 3.4). This implies that the increase of hemolymph ecdysteroids is a slow process, taking more than 2 days to raise ecdysteroid levels significantly.

LN ticks, which were not at the threshold levels of ecdysteroid titer for salivary gland degeneration to occur (30 ng/mL, Harris and Kaufman, 1985), and whose ecdysteroid levels did not change, showed a significant decrease in secretion (Fig. 3.3). As in Chapter Two, this appears to represent the component of fluid secretion seen in below critical weight ticks which is recoverable and which does not represent true degeneration.

It should be noted that the nitrogen inflated ticks showed high hemolymph ecdysteroid titers on both day 0 and day 2, well within the previously established range of titers correlated with salivary gland degeneration (Harris and Kaufman, 1985, Kaufman, 1991). In view of this, the elevated fluid secretory rate for both SINF and LINF ticks on days 0 and 2 was surprising. In fact, LINF ticks had day 2 secretory rates that were almost higher than at day 0 ($p = 0.058$). AN control ticks, which normally degenerate within four days of removal from the host (Harris and Kaufman, 1984), did not show a decrease in secretion by day 2 either. This observation is very surprising in light of previous work and I have no obvious explanation for this discrepancy. Other work has shown that at least some autophagic vacuoles appear in the secretory labyrinth within 24 hours of removal from the host in replete ticks, although significant reduction in secretory competence occurs by day 2 (Harris and Kaufman, 1984). Long incubation times with ecdysteroids appear to be required before there is physiological evidence of degeneration, and so loss of function of the glands may not imply ultrastructural degeneration.

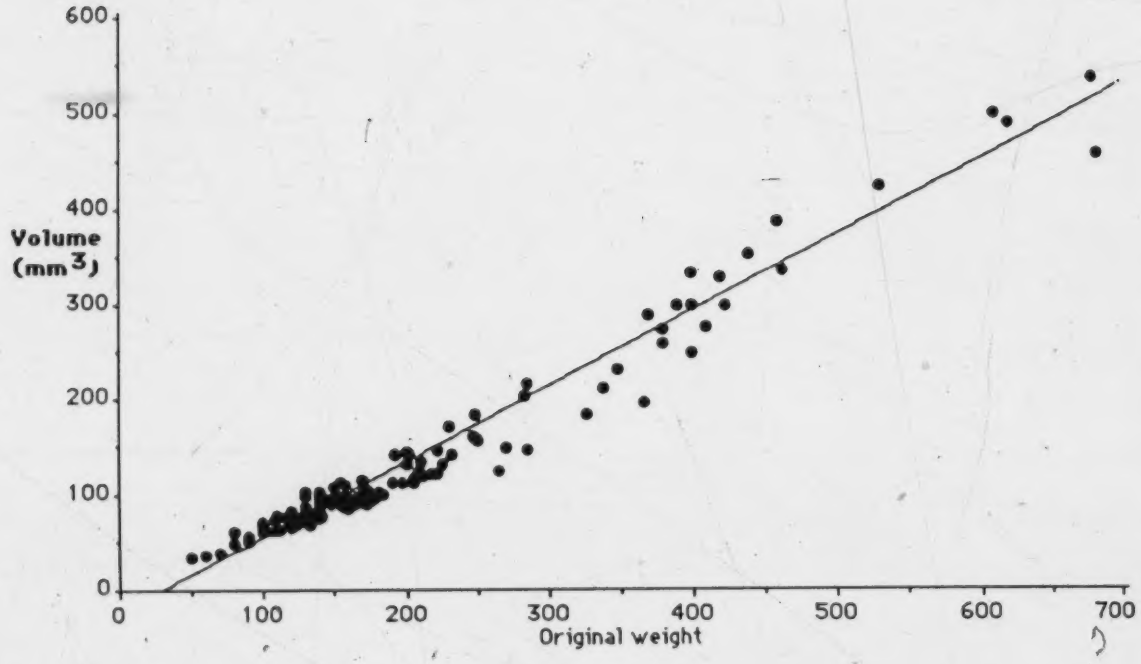

Fig 3.1: Volume of *A. hebraeum* normally-fed ticks plotted as a function of weight (linear regression line of y= .836x - 24.113, with an R^2 of 0.988). Volume was calculated from the formula for a cylinder (V = 4/3 π abc) which roughly approximates tick shape.

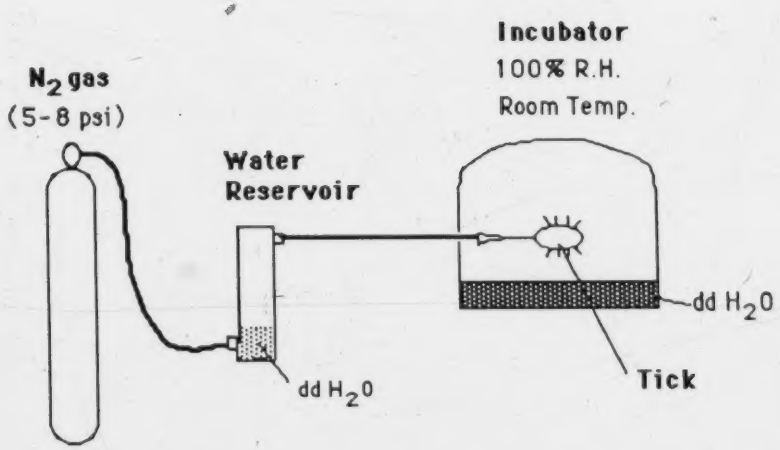

Fig. 3.2: Schematic diagram showing the air infusion apparatus. For detailed description of methods, see text. (ddH_2O: double distilled water)

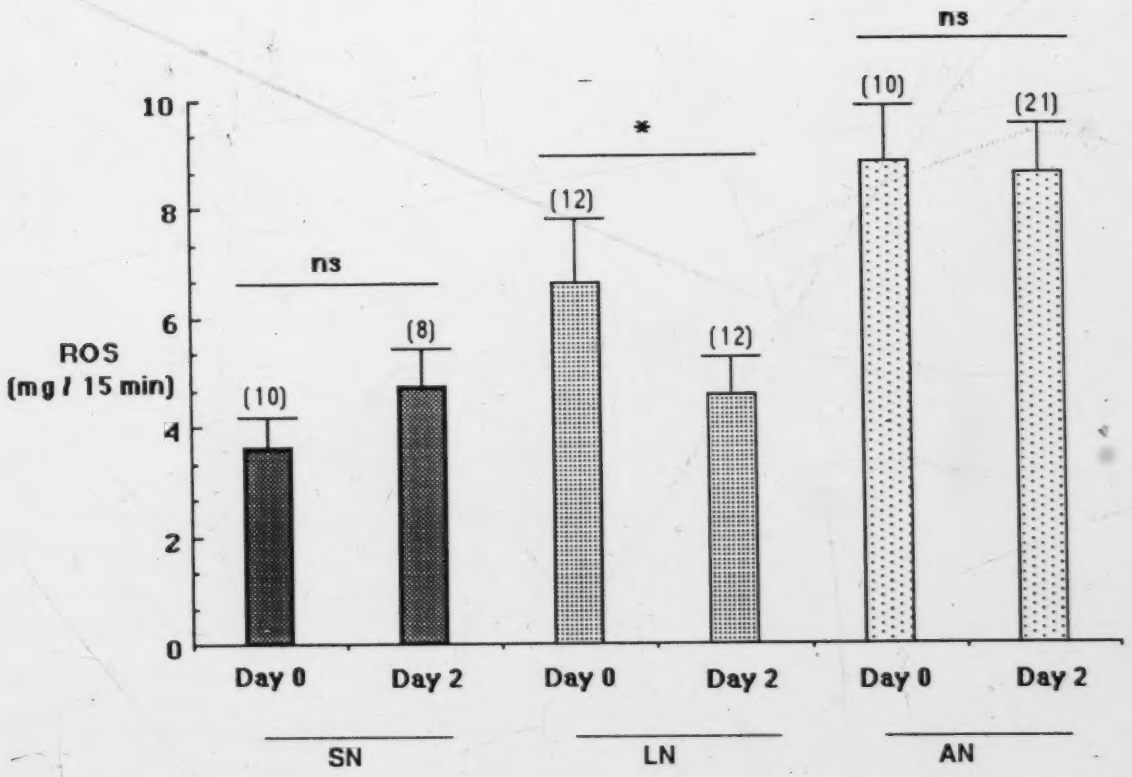

Fig. 3.3: Rate of secretion for salivary glands of normally-fed *A. hebraeum* ticks on day 0,and after two days of incubation (day 2). Means and standard error are shown. Significance is indicated for each weight class (as defined in text)(ANOVA; ns: $p>0.05$, *: significant, $p < 0.05$). Sample size is shown in parentheses. Abbreviations as in text.

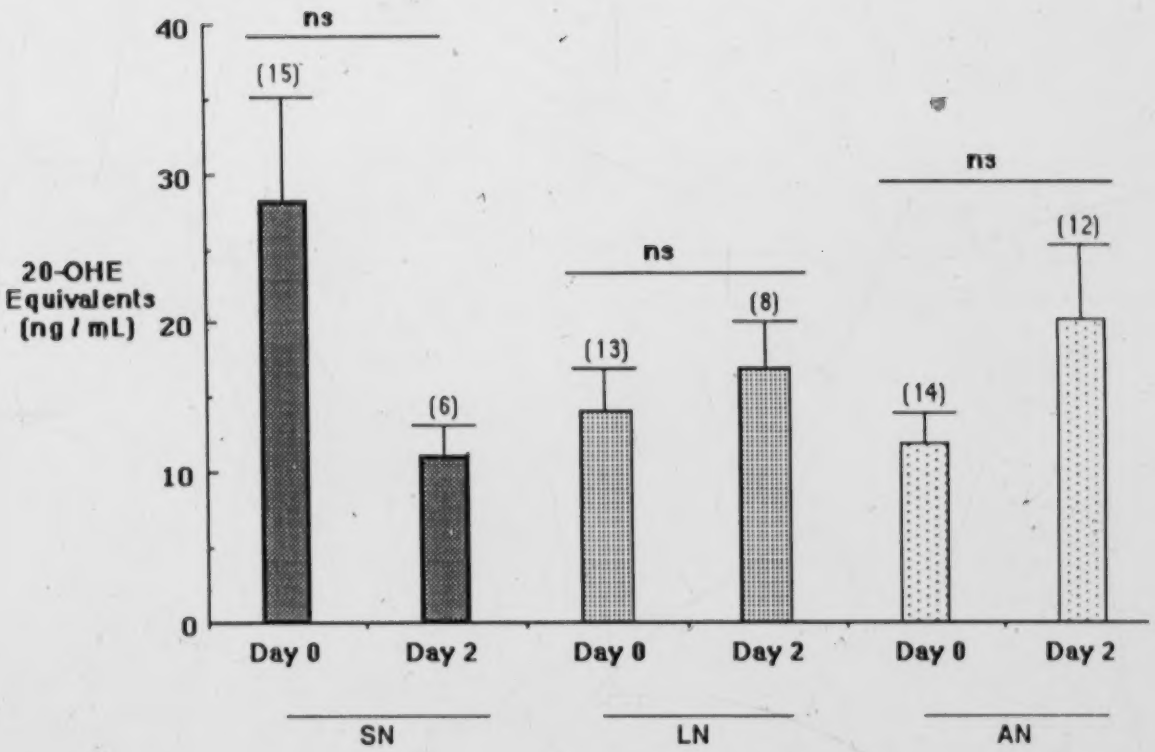

Fig. 3.4: Hemolymph ecdysteroid levels in normally-fed *A. hebraeum* ticks immediately after, and 2 days after removal from the host (day 0 and 2). Means and standard error are shown, and sample sizes are shown in parentheses. Significance is indicated (ANOVA, ns: $p>0.05$). Abbreviations as in text.

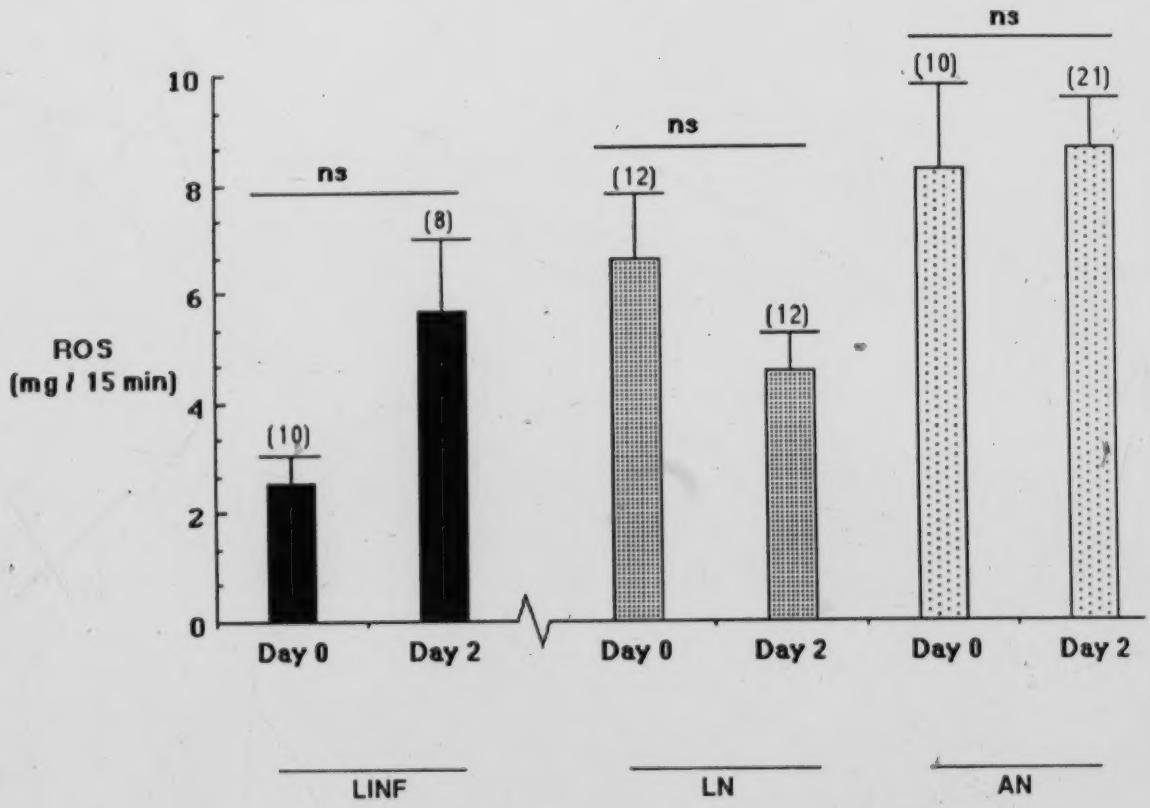

Fig. 3.5: Rate of secretion of N_2 infused LINF and normally-fed *A. hebraeum* ticks immediately after, and two days after infusion or removal from the host (day 0 and 2). Means and standard error are shown. Significance is indicated for each weight class (ANOVA, **ns**: $p>0.05$). Sample size is shown in parentheses. Data for LN and AN ticks are taken from Fig. 3.3.

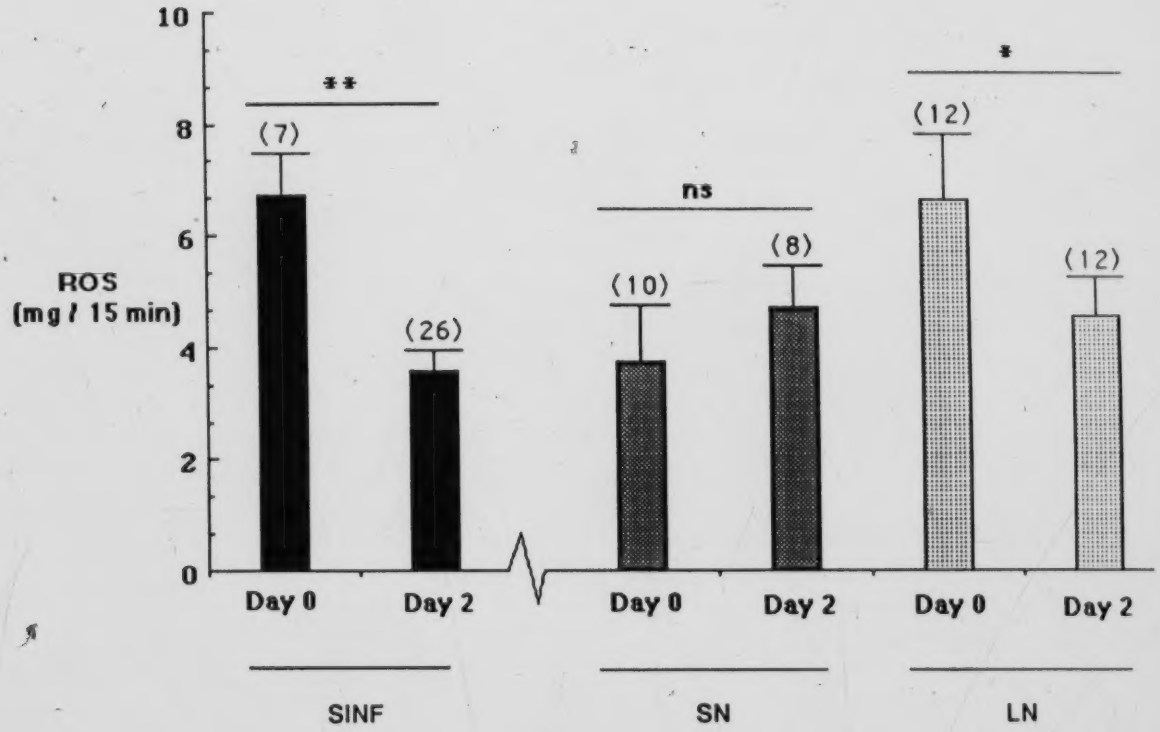

Fig. 3.6: Rate of secretion of N_2 infused SINF and normally-fed *A. hebraeum* ticks immediately after, and two days after removal from the host (day 0 and 2). Means and standard error are shown. Significance is indicated for each weight class (as defined in text) (ANOVA; **: $p < 0.01$; *: $p < 0.05$; **ns**: $p>0.05$). Sample size is shown in parentheses. Data for SN and LN ticks are taken from Fig. 3.3.

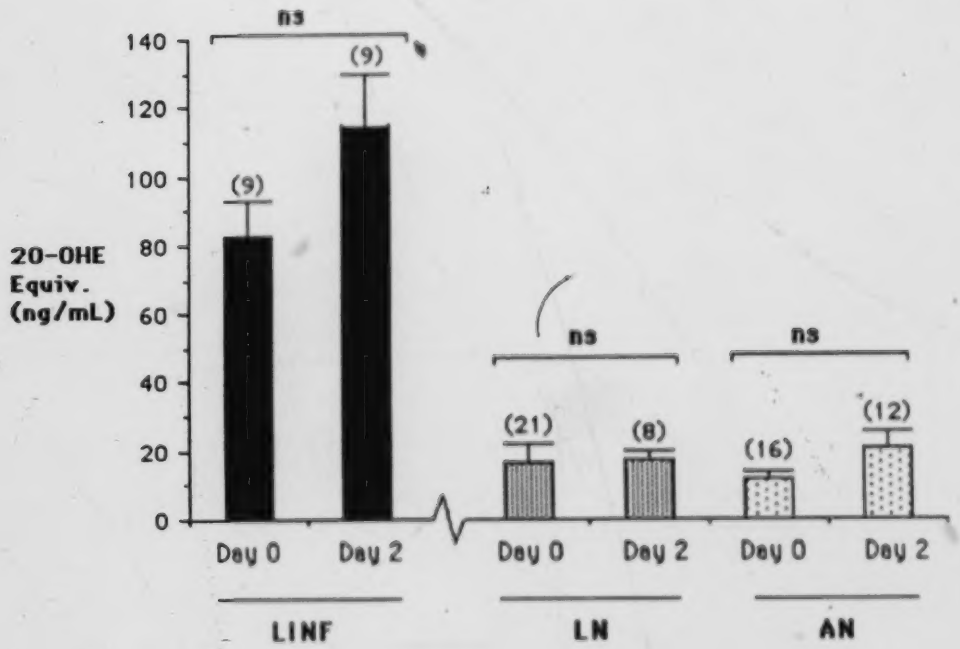

Fig. 3.7: Hemolymph ecdysteroid levels in N_2 infused LINF and normally-fed control *A. hebraeum* ticks immediately after, and two days after infusion or removal from the host (day 0 and 2). Means and standard error are shown. Significance for each weight class is indicated (ANOVA, ns: $p>0.05$). Abbreviations as in text. Sample size is shown in parentheses. Data for LN and AN ticks are taken from Fig. 3.4.

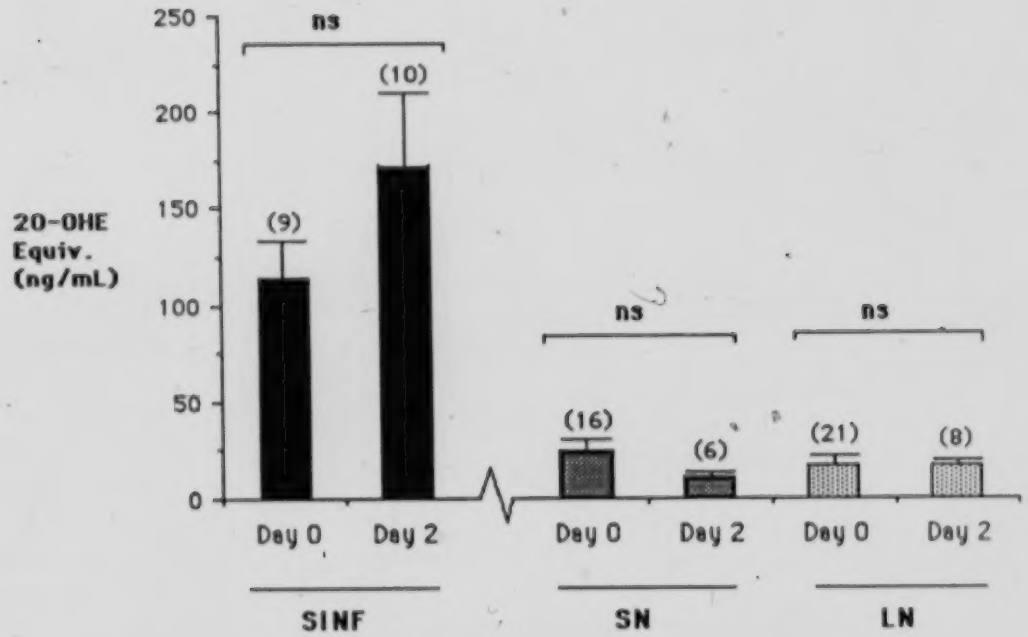

Fig. 3.8: Hemolymph ecdysteroid levels in N_2 infused SINF and normally-fed *A. hebraeum* ticks immediately after, and two days after infusion or removal from the host (day 0 and 2). Means and standard error are shown. Significant differences between weight classes are indicated (ANOVA; **ns**: $p>0.05$). Abbreviations are indicated in text. Sample size is indicated in parentheses. Data for SN and LN ticks are taken from Fig. 3.4.

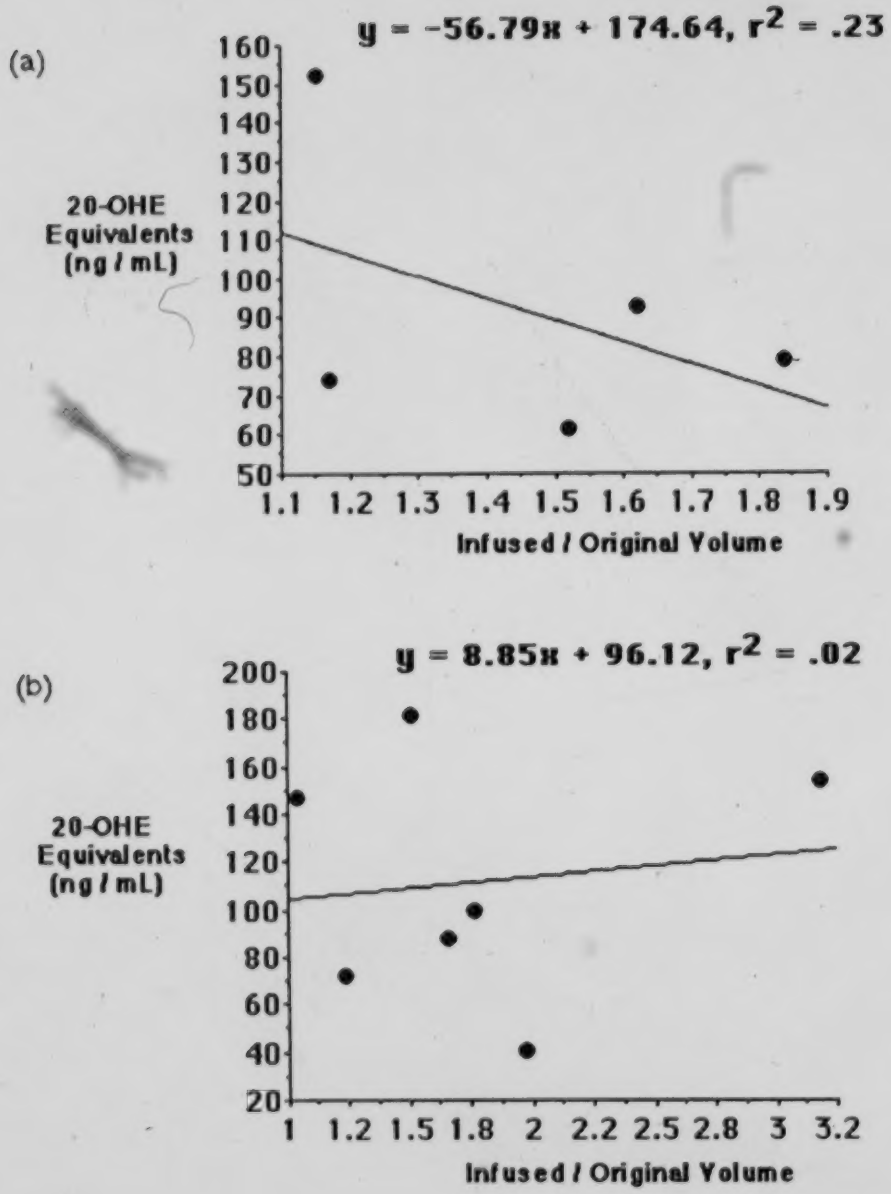

Fig. 3.9: Hemolymph ecdysteroid levels as a function of the ratio of infused to original body volume in LINF *A. hebraeum* ticks (a) immediately after (day 0) and (b) two days after infusion (day 2). Data are taken from Fig. 3.7.

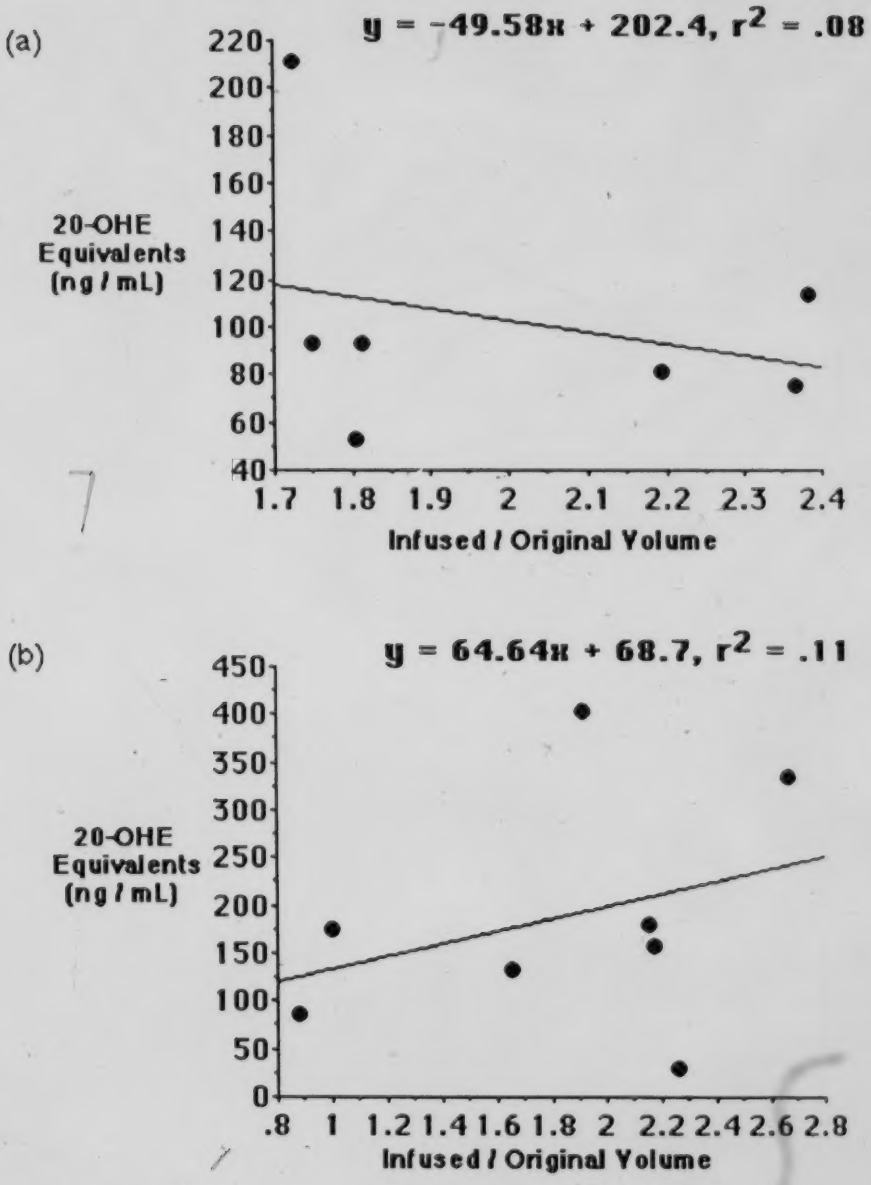

Fig. 3.10: Hemolymph ecdysteroid levels as a function of the ratio of infused to original body volume in SINF *A. hebraeum* ticks (a) immediately after (day 0) and (b) two days after infusion (day 2). Data are taken from Fig. 3.8.

Chapter Four

The Effect of Ecdysteroids on Other Aspects of Tick Biology

Introduction

Ecdysteroids are probably involved in physiological events other than just salivary gland degeneration. There have been reports suggesting involvement in vitellogenesis, egg development (Connat, *et al*, 1987) and moulting (Germond, *et al*, 1980, Delbecque, *et al*, 1978). Ecdysteroids may also stimulate synthesis of neurosecretory material synthesis in cells of the CNS (Khalil, *et al*, 1988) and may stimulate pheromone release (Sonenshine, 1985). To further investigate other areas of possible influence by ecdysteroids, degeneration in a diapausing tick and reattachment to the host after degeneration of the salivary glands were examined, for reasons explained below.

Degeneration in a diapausing tick

Certain species of ticks undergo a morphogenic diapause, halting oviposition presumably to avoid unfavorable environmental conditions (Belozerov, 1982). Oocyte development is generally uncoupled from digestion during diapause; it has been suggested that digestion is not halted, but rather, uptake of protein from the hemolymph by the ovary is probably reduced (Belozerov, 1982).

Ecdysteroid titers and the state of the salivary glands in diapausing ixodid ticks are not known, although ecdysteroids have been suggested to be involved in breaking diapause and initiating vitellogenesis in some tick species. However, there appear to be major differences between ixodid and argasid ticks with respect to hormonal involvement in breaking diapause and initiating vitellogenesis. Ecdysone and 20-OHE induce a break in diapause, initiating vitellogenesis, in two ixodid ticks, *Dermacentor albipictus* and *Rhipicephalus sanguineus* (Wright, 1969; Sannasi and Subramoniam, 1972), but 20-OHE inhibits vitellogenesis in argasid ticks (Diehl, *et al*, 1982b). Juvenile hormone

breaks diapause and initiates vitellogenesis in several adult argasid species (Bassal and Roshdy, 1974; Diehl, *et al*, 1982; Connat, *et al*, 1983).

Ecdysteroids may be involved in salivary gland degeneration and other processes such as vitellogenesis in ixodid ticks but, since these events are closely linked chronologically, it is difficult to demonstrate these effects independently from each other. However, a diapausing tick, such as *H. anatolicum excavatum* had potential for providing further evidence that TSGDF is an ecdysteroid. If salivary gland degeneration is inhibited during diapause, one would expect that condition to be correlated with low hemolymph ecdysteroid titers. Thus hemolymph ecdysteroid levels and vitellin uptake by ovaries were measured to investigate salivary gland degeneration, hemolymph ecdysteroid titer and vitellogenesis during and after diapause. It was hoped that studying these ticks through diapause would help to distinguish the effect of ecdysteroids on salivary gland degeneration isolated from their possible involvement in vitellogenesis.

Ecdysteroids and reattachment to the host

Ticks below the critical weight lose some fluid secretory competence four days after removal from the host, but can recover the lost function if allowed to reattach to the host (Harris and Kaufman, 1984). Ticks allowed to feed to repletion also lose secretory function four days after dropping off the host, but it is not known if this is recoverable, as engorged ticks will not reattach to the host. As ecdysteroid titers increase in engorged ticks after dropping off the host (Connat, *et al* , 1985, Kaufman, 1991), it is possible that ecdysteroids may directly inhibit reattachment to the host, in addition to causing salivary gland degeneration. By injecting small partially fed ticks under the critical weight with 20-OHE to induce salivary gland degeneration, and giving these ticks access to the host, the effect of ecdysone on the reattachment and feeding process was tested.

Materials and Methods

Ticks

For the diapause experiment, adult *Hyalomma anatolicum excavatum* ticks provided by Professor W. Knülle (Frei Universität Berlin, FRG) were allowed to feed to engorgement on rabbits in September and October of 1989, and were held in incubation at 26 °C, 95% RH, prior to analysis with the vitellogenesis assay described below.

Amblyomma hebraeum ticks were taken from our laboratory colony raised as described in Chapter 2 for use in both the diapause and ecdysteroid-infusion experiments.

Drugs and Media

20-OHE was dissolved in 70% ethanol at a concentration of 12 mg/mL, and diluted 200-fold in sterile TC199 to 60 µg/mL, for use in the ecdysone injection experiment. Ethanol control solutions were at a concentration of 0.35% ethanol diluted in sterile TC199.

Vitellogenesis Assay

Ovaries and eggs collected during and after diapause were analyzed for vitellin content in a spectrophotometric assay, as described by Kaufman *et al* (1986). Vitellin has a prosthetic haem group which allows changes in concentration to be detected by optical density. Ovaries of *Hyalomma anatolicum excavatum* and *Amblyomma hebraeum* ticks were dissected out in Hank's medium, blotted, weighed and homogenized in 3.4 mL of distilled water. Egg samples of both species were prepared in the same manner. The homogenates were frozen until analyzed, when they were centrifuged at 20,000 G for 15 minutes prior to spectrophotometric measurement. A Beckman DU-8 spectrophotometer was used to measure the optical density of centrifuged ovary and egg extracts at a wavelength of 400 nm (specific for haem), and at 500 nm (background). Vitellin

content is expressed as the absorbance at 400 nm minus that at 500 nm per gram ovary (or egg) weight.

Ecdysone Infusion

For the ecdysone infusion experiment, the ticks were infused through the camerostomal fold with 20-OHE or ethanol vehicle for 24 hours on a Harvard syringe pump at a rate of 2.4 +/- 0.2 (9) µL/hr (total 20-OHE = 3.5 µg/24 hrs.). Sterile all-glass syringes with 30-gauge needles were used for infusion. Prior to an experiment the pump was run for at least 15 min to ensure that a constant rate of flow was reached before the experiment began.

Degeneration in a diapausing tick

To determine the timing for salivary gland degeneration and vitellogenesis in a diapausing tick, engorged *H. a. excavatum* were incubated in darkness at 26°C for a two to three month period, and periodically sampled to monitor progression of salivary gland degeneration and hemolymph ecdysteroid concentration. Due to the limited numbers available, and since it was not known if, or when salivary gland degeneration took place in relation to vitellogenesis, only three to five ticks were sampled at approximately three to four week intervals, so as to allow sampling to continue until oviposition.

After diapause had broken and egg-laying initiated, samples of hemolymph, ovary and egg were taken for analysis. One sample was also collected 7-8 days after initiation of oviposition.

For comparison, samples of hemolymph, ovary, and egg were taken from *A.hebraeum* for ecdysteroid and vitellin content, and the salivary glands were assayed for secretory competence, immediately after engorgement and during egg-laying.

Ecdysteroids and reattachment to the host

A small partially-fed tick can regain salivary gland function lost following removal from the host provided it can reattach to the host (Harris and Kaufman, 1984). It has been suggested that down-regulation or uncoupling of the dopamine receptor and second messenger system may cause this decrease in function (Kaufman, 1986), rather than TSGDF-induced degeneration. Ticks above critical weight, however, will not reattach. To test whether ecdysteroids also inhibit reattachment and feeding, partially fed ticks, below the critical weight, were infused for 24 hours with 20-OHE. One group was infused with 60 µg/mL 20-OHE, and the other with the ethanol vehicle (0.35%) in sterile TC199, following an initial priming dose of 20-OHE or ethanol (2 µL/100 mg body weight, 0.12 µg 20-OHE/100 mg body weight) with an 'Agla' micrometer syringe apparatus (Wellcome Reagents Ltd.). A 24 hour infusion time was necessary since a single bolus injection is not effective in inducing salivary gland degeneration (Kaufman, unpublished). Five days after removal from the infusion apparatus, when degeneration of the glands should have been complete, six to eight ticks from each treatment were assayed for fluid secretory competence and hemolymph ecdysteroid titer, and the remaining 15 to 20 infused ticks were returned to the host to reattach and feed. If the ticks did reattach and feed, their secretory competence and hemolymph ecdysteroid levels were measured, after they had fed to repletion and dropped off the host.

Statistics

Results are reported as mean and SEM (n). All statistics were done with both Statview 512 (Abacus Concepts, CA) on a Macintosh SE microcomputer and the MIDAS statistical package of the University of Alberta mainframe computer.

Results

Degeneration during diapause

Fluid secretory competence had fallen almost to zero by 17 days post-engorgement, when the tick had already entered diapause, and remained at this low level throughout diapause (Fig. 4.1). Hemolymph ecdysteroid titer, although very low throughout diapause, was highest at day 17 (approximately 7 ng 20-OHE equiv./mL hemolymph), when the first assay was done, but fell by day 41 to approximately 2.5 ng 20-OHE equiv/mL hemolymph and remained at this level throughout the rest of the sampling period (Figs. 4.1 and 4.2). Vitellin content of the ovary appeared to rise well before oviposition, with a large increase occurring 25 days prior to oviposition (day 72 postengorgement, Fig. 4.2). Vitellin content remained at this level throughout oviposition.

Ecdysteroids seemed not to be very much concentrated in the egg, relative to the ovary in *H. excavatum* (Fig. 4.3). Hemolymph ecdysteroid levels, however, were much lower than both ovary and eggs, on day 1 of oviposition (1 ng/mL for hemolymph vs about 50 and 70 ng/mg for ovary and egg, respectively).

Ecdysteroids and reattachment to the host

Reattachment to a rabbit host after infusion with 20-OHE is shown in Table 4.1. Of the ticks infused with 20-OHE, only 15% reattached to the host and the remainder either failed to attach or died, unattached, on the host. Only forty-four percent of the ethanol controls reattached, a percentage nevertheless significantly different from that of the experimental ticks (Chi-squared test, $p<0.05$).

Table 4.1: The frequency of reattachment of *A. hebraeum* ticks infused for 24 hours with 20-OHE. The asterisk indicates a significant difference between ecdysone infused and control ticks' reattachment ratios (Chi-squared test, $p<0.05$).

Treatment	Attached	Unattached	TOTAL
20-OHE infused	3	17	20
EtOH controls	7	9	16

Fluid secretory competence for the 20-OHE infused ticks was reduced by 80% by day 5 of incubation (Student's t-test, $p < 0.05$), compared to ethanol controls (Table 4.2). Hemolymph ecdysteroid levels were almost 10 times the control hemolymph levels (122 ng/mL vs 13 ng/mL; Student's t-test, $p < 0.05$, Table 4.2).

The few ticks that did reattach to the host showed some surprising results in terms of both secretory competence and hemolymph ecdysteroid levels. Secretory competence rebounded to approximately the control levels (Table 4.2), while hemolymph ecdysteroid levels decreased to near the control levels, a 67% decrease from five day postincubation ecdysteroid titers. Neither secretory rate nor hemolymph ecdysteroid levels were significantly different from those of controls.

Table 4.2: Rate of fluid secretion and hemolymph ecdysteroid titers of 20-OHE infused *A. hebraeum* ticks incubated for five days or allowed to reattach to naive rabbit hosts. Mean and standard error are shown, with sample size in parentheses. Significant differences between infused ticks and controls are as indicated (t-test, *: $p < 0.05$, ns: $p<0.05$).

Treatment	ROS (mg/15 min)		[20-OHE] (ng/mL)	
5 days postincubation				
Ecdysone infused	0.40 +/- 0.20 (15)	*	122 +/- 47 (8)	*
EtOH controls	2.08 +/- 0.15 (11)		13 +/- 1.4 (6)	
Reattached ticks				
Ecdysone infused	7.72 +/- 0.88 (6)	ns	40 +/- 14 (3)	ns
EtOH controls	9.72 +/- 2.28 (10)		22 +/- 2.4 (5)	

Discussion

Degeneration in a diapausing tick

The low sample sizes for this experiment made it difficult to draw many conclusions from the data, but some trends were apparent. Degeneration of the salivary glands appeared not to be correlated with high ecdysteroid titer in the diapausing ticks, unlike previous work correlating increased ecdysteroid titers with degeneration. Fluid secretory rates remained close to zero throughout diapause, and the glands looked quite degenerated by day 17, but hemolymph ecdysteroid titers were extremely low throughout diapause (Fig. 4.1). However, ecdysteroid levels may have been higher prior to day 17 when the earliest sample was taken. The decrease in ecdysteroid titer from day 17 to day 41 (7 ng/mL to 2.5 ng/mL, Fig. 4.1 and 4.2) may be the end of a falling trend in ecdysteroid titer. Unfortunately, there were not sufficient ticks to sample during the immediate postengorgement period and so the question of whether ecdysteroids induce degeneration in diapausing ticks cannot be answered with this data.

Ecdysteroids did not appear to be involved in the initiation of vitellogenesis, as has been suggested to occur in insects (Hagedorn, 1983), supporting other, more direct, evidence from our laboratory (Lunke and Kaufman, in preparation). Ovary vitellin increased and hemolymph ecdysteroid titers decreased during diapause: ovary vitellin content doubled from day 17 to day 72 postengorgement (Fig. 4.2).

Ecdysteroid titers were much higher in eggs and ovary than hemolymph (70 and 50 times higher respectively), similar to the trend in *A. hebraeum* , although in the latter species, the ovary's ecdysteroid level is only about four times the hemolymph titer (Kaufman, 1991). There are several possible explanations for the concentration of ecdysteroids in the ovary and eggs: (1) ovary and eggs accumulate ecdysteroids much earlier in diapause, before hemolymph levels fell to the low levels observed during the sampling period in this experiment, and (2) the ovary itself synthesizes the ecdysteroids.

The first explanation seems possible, but this is dependent on there being higher ecdysteroid concentrations in the first seventeen days following engorgment, a period for which I have no data, but for which there is evidence supporting this slower pattern of accumulating ability for the ovary (Connat, *et al*, 1985; Kaufman, 1991). The last explanation is also possible, especially considering the magnitude of the ecdysteroid gradient. Ecdysteroid synthesis is suspected in tissues such as the fat body (Schriefer, *et al*, 1987; Ellis and Obenchain, 1984), and CNS (Soneshine, *et al*, 1985). No evidence for synthesis in the ovary yet exists. Also, although the ovary has been found to accumulate ecdysteroids, it does so after a delay, and follows an increase in the whole carcass of the tick (Connat, *et al*, 1985), suggesting it does not synthesize ecdysteroids itself. Thus, further study is required before the explanation for the large difference between hemolymph and ovarian ecdysteroid titer will become clear.

Ecdysteroids and reattachment to the host

Infusion of 20-OHE inhibited the percentage of small partially-fed ticks which reattached. After being on the host for 11 days, 85% of the ticks did not attach or died, unattached, on the host. Hemolymph ecdysteroid levels were within the physiological range for salivary gland degeneration and the salivary glands were functionally degenerate after five days of incubation. Thus, salivary gland degeneration and high ecdysteroid levels seem to have inhibited reattachment. The controls, which had the low levels of ecdysteroids and secretory competence characteristic of small partially-fed ticks remaining off the host (Harris and Kaufman, 1984) appeared to be in normal physiological condition. However, since they also had a low reattachment ratio (about 44%), it may be that ethanol also inhibits reattachment.

Reattached ticks had decreased levels of ecdysteroids and recovered secretory competence, suggesting that they had recovered from the stimulated degeneration. The salivary glands of the reattachees, although somewhat smaller than normal, appeared

healthy. There were only a limited number of reattachees (15% of the infused ticks) however. Reattachment could be due to misinjection of the ticks, and nonsufficient salivary gland degeneration prior to reexposure to the host. The unattached ticks could not be assayed for secretory competence or ecdysteroid titer for comparison, most of them being quite dehydrated by the last day on the host. The glands of ticks injected with ecdysone examined after five days incubation were in very poor condition, however, and were very fragile and degenerated. Perhaps in certain circumstances degeneration is recoverable.

Attachment of ticks to the host probably involves olfactory, tactile and temperature stimuli (Balashov, 1972), and these factors will undoubtedly affect reattachment, in addition to the condition of the salivary glands. The question is whether the ticks were able to interpret these cues normally. Ecdysteroid's effects have been seen in salivary glands, cuticle and moulting activity of the ticks (Harris and Kaufman, 1985; Soloman, *et al*, 1982; Diehl, *et al*, 1982a; Germond, *et al*, 1980; Delbecque, *et al*, 1978), but other effects on the tick are not well understood. It is possible that elevated ecdysteroids could interfere with perception of environmental stimuli. Ecdysone has been suggested to cause an increase in neurosecretory material in cells of the CNS of the ixodid tick, *Hyalomma dromedarii*, but the functional effect of these changes is not known (Khalil, *et al*, 1988). And, it has also been shown to stimulate sex attractant pheromone activity and release in ixodid ticks (Sonenshine, 1985). Thus, although it appears 20-OHE was able to inhibit reattachment, this experiment did not determine whether ecdysteroid's effects on reattachment were an indirect consequence of salivary gland degeneration. It is possible that the increased ecdysteroid titers blocked reattachment through some other pathway.

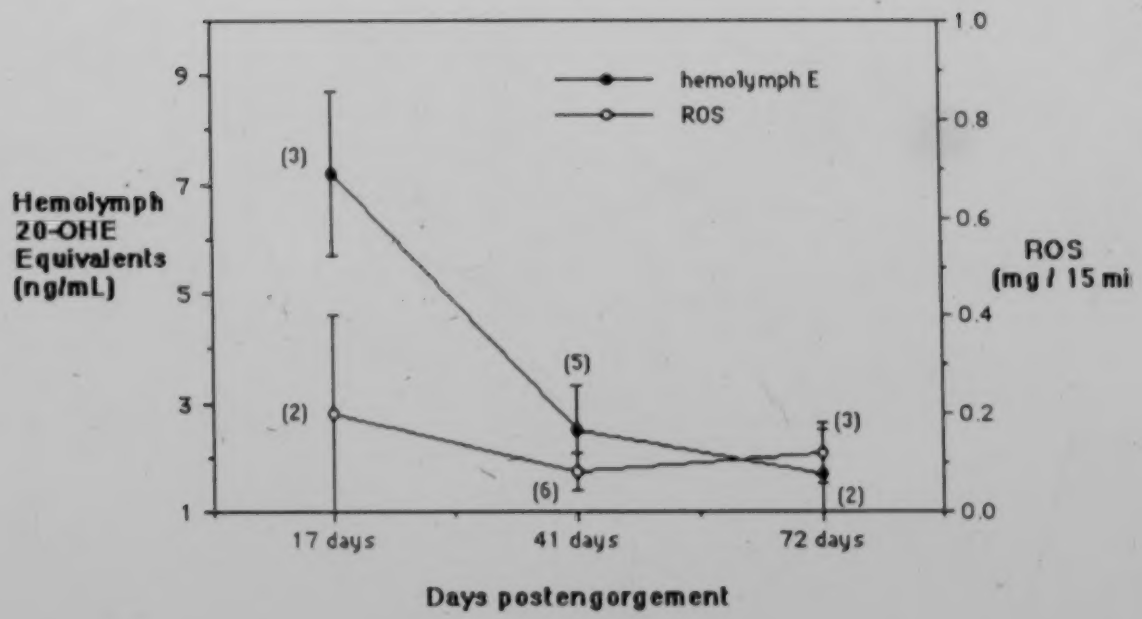

Fig. 4.1: Hemolymph ecdysteroid titers and fluid secretory competence (ROS) of salivary glands of female *Hyalomma excavatum* ticks during the diapause following engorgement. Mean and standard errors are shown, with sample size shown in parentheses.

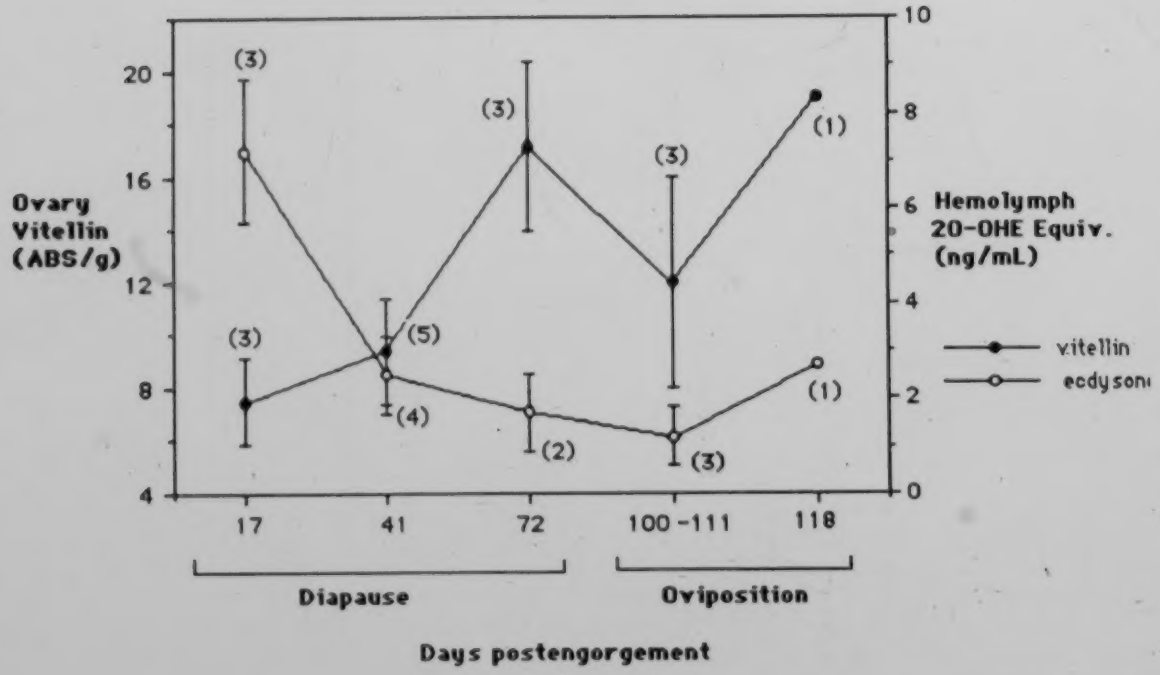

Fig. 4.2: Ovary vitellin titers (absorbance at 400 nm/gram tissue) and hemolymph ecdysteroid titers (ng/mL) in *Hyalomma excavatum* ticks during diapause and oviposition. Mean and standard error are shown. Sample size is shown in parentheses. Data for hemolymph ecdysteroids during diapause is taken from Fig. 4.1.

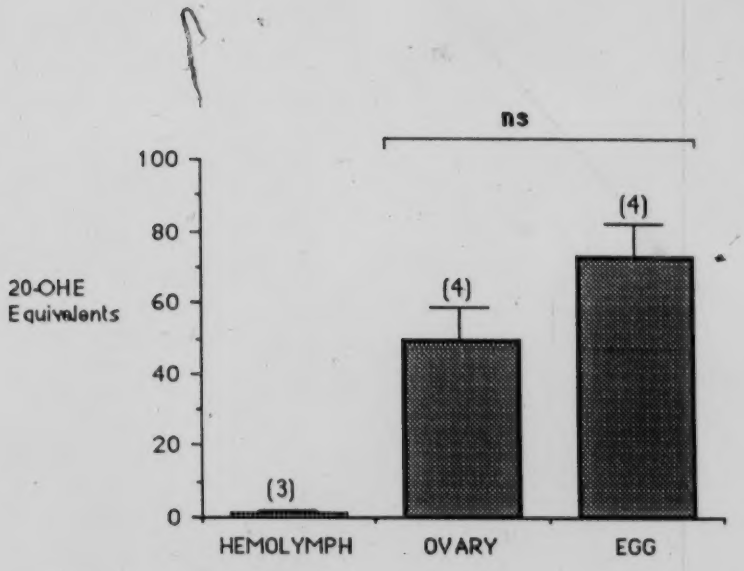

Fig. 4.3: Tissue ecdysteroid titers on day one of oviposition for *Hyalomma excavatum*. Mean and standard error are shown. Sample size is shown in parentheses. Significance is indicated with **ns** (nonsignificant, ANOVA, $p > 0.05$). 20-OHE is in units of ng/mL for hemolymph, ng/g for egg and ovary.

CHAPTER FIVE

General Conclusion

Attempts to stimulate release of ecdysteroids and salivary gland degeneration by liquid and nitrogen gas infusion failed to meet the criteria of the experimental protocol. Reduction in salivary fluid secretory rate was not correlated to an increase in hemolymph ecdysteroid titers, and neither hemolymph ecdysteroid titer nor fluid secretory rate were correlated to a net increase in body weight due to infusion. These factors should be correlated if salivary gland degeneration is regulated by abdominal stretch.

Some of the data suggests that the failure to induce salivary gland degeneration may have been due to the method of inducing stretch, rather than the basic hypothesis being false. The general difficulty in infusing LINF ticks to beyond the critical weight, * and the toxic effects of infusing ticks at a rapid rate (33 µL/hr) indicate that the technique may have been too stressful for the ticks. Also, the experiments with nitrogen gas seemed to dry out the ticks. Nijhout (1979) succeeded in inducing moulting in some individuals by infusion of both saline and air into *Oncopeltus fasciatus*. However, similar attempts with *Rhodnius prolixus*, which has been shown to have stretch-mediated release of ecdysone by other, mainly neurophysiological, means (reviewed in Wigglesworth, 1970), failed to induce hormone release and moulting (Nijhout, 1981). Because of the generally toxic reaction to stretch induced by infusion of liquid and inflation with nitrogen gas in ticks, the question remains as to whether abdominal stretch stimulates salivary gland degeneration. Because the control ticks had reduced secretory competence and increased ecdysteroid titers at a common critical weight, this still suggests that body size may be the key factor triggering the degeneration response. It remains to be shown whether abdominal stretch mediates this response.

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* Most of the LINF ticks were infused to a level not much above the critical weight.

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APPENDIX A

Composition of Hank's and TC199 media.

Hank's medium

Compound	wgt/L (g/L)
NaCl	11.5
D-glucose	1.6
KCl	0.4
$CaCl_2$	0.14
$MgSO_4$	0.0098
$KH(2)PO_4$	0.06
$NaHPO_4$	0.05
phenol red	0.01

-360 mOsm
- pH brought to 7.2 - 7.3 with NaOH

TC199 medium

-one package of powdered Medium 199 (Gibco Laboratories, Cat. # 400-1200)
-2.1g NaCl
-2.09g 3-(N-morpholino) propanesulfonic acid (MOPS; Sigma Chemical Co.)
-dissolved in deionized water
-pH adjusted to 7.2 - 7.3 with NaOH and final volume brought to 1.0 L
360 mOsm